FlowJo

Data Analysis Software for Flow Cytometry

Advanced Tutorial

FlowJo was written by Adam Treister and Mario Roederer beginning in 1996, based on concepts developed at the Herzenberg laboratory at Stanford.
We are indebted to our active and enthusiastic users worldwide for their ideas, discussions and tireless testing of new versions.

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• FlowJo Advanced Tutorial •
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Revision Date: **February** 22, 2010
Version 7.6

FlowJo Tutorial

FlowJo is a software application designed to create an integrated environment for viewing and analyzing flow cytometric data. This environment is presented in the form of a ***Workspace***. The Workspace contains a list of all of the data samples that you load, the gates, statistics and other analyses that you apply, and the table templates and graphical layout templates that you design. The Workspace is saved as a FlowJo document on your hard disk; when you re-open the document, you will see the status of your analyses as they were when you last saved the Workspace.

This tutorial is designed to introduce you to the program. Reading through it, you will learn how to operate FlowJo. Run the program as you perform the steps in the tutorial so that you can get the best feel for how the program works. As you watch FlowJo perform various operations such as creating new graphs, statistics, tables, or graphical layouts, you will see how fast and easy FlowJo is to use. It will take you three to six hours to complete the tutorial (you can easily break it up by chapter).

The tutorial is designed around an example data set. The experiments used for the tutorial are based on 3-color immunophenotyping of human peripheral blood mononuclear cells (PBMC). The steps shown in this tutorial will help you in the analysis of nearly any kind of FACS data.

The tutorial is only an introduction! FlowJo is capable of much that simply can't be covered in an introduction such as this (for example, there are Analysis Platforms to perform sophisticated DNA/Cell Cycle analysis, Kinetics analysis, Compensation, etc).. You can learn more about FlowJo, and in particular how to use these platforms, through the on-line help facility. Whenever you ask for help from FlowJo, it launches a web browser and accesses help pages about the topic you selected. You can navigate the help pages to find out more about all aspects of FlowJo. To access this on-line user manual directly, point your browser to http://www.flowjo.com/v76/en/windowstoc.html

The online help documentation provides tutorials for Compensation, Kinetics, and DNA/Cell Cycle analysis; in addition, Tree Star provides Demonstration Data to let you explore these platforms. http://www.flowjo.com/home/tutorial.html

In addition, there is a web page for FlowJo FAQs (Frequently-Asked Questions). http://www.flowjo.com/home/faq.html . Here you may find the answers to a problem you have had. FAQs are also instructive; you may learn new techniques from reading them. You may also have a look at our blog, the ***Daily Dongle,*** for the latest Flow issues or questions. (http://flowjo.typepad.com/)

Finally, we are pleased to be able to frequently update FlowJo to provide new features and analysis capabilities. **Therefore, it is possible that the graphics shown in this tutorial may not exactly match the windows that you see when you run the most recent version of FlowJo.** You can always download the most recent version of FlowJo from the web site listed above.

Table of Contents

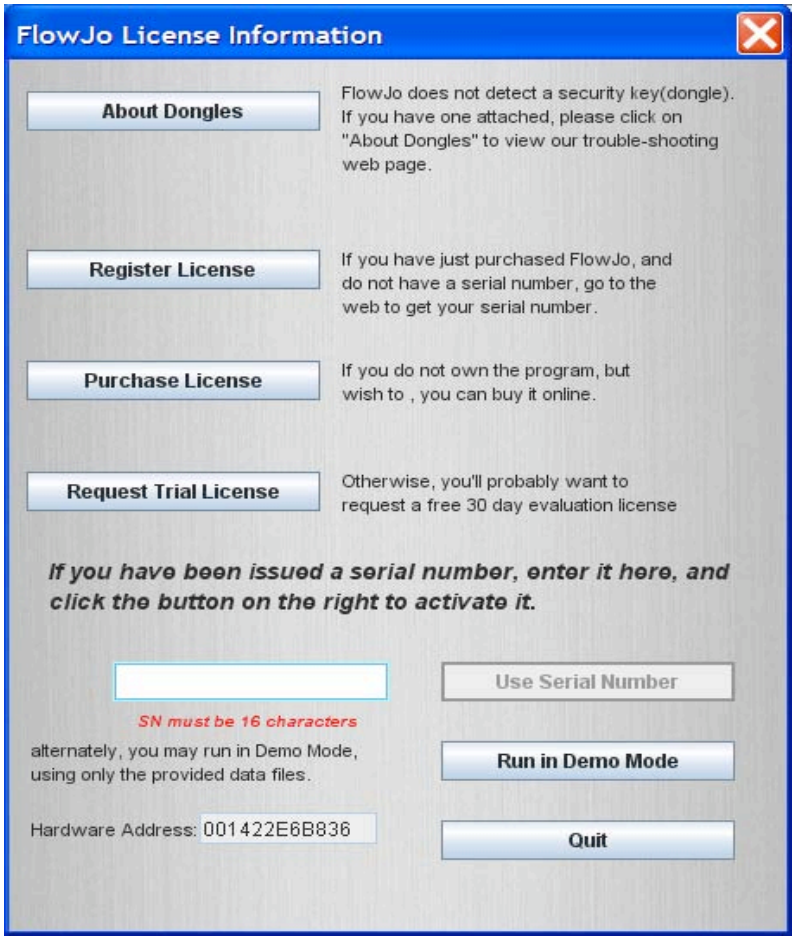
Introduction 6
Lesson 1: Workspaces and Basic Data Display 11
Lesson 2: Gating and Statistics 18
Lesson 3: Copying Analyses to Other Samples 26
Lesson 4: Groups and Batch Analyses 30
Lesson 5: Modifying Group Analyses 36
Lesson 6: Tables and Layouts – Collating Data Output 44
Lesson 7: Creating Simple Graphical Layouts 50
Lesson 8: Creating Batch Graphical Reports 63
Lesson 9: Generating Complex Batch Analysis 70
Lesson 10: Creating Finished Reports 77

Introduction

This chapter is designed only to give you a quick overview of the powerful batch features that FlowJo provides – don't worry about trying to learn how to use the program during this demonstration. Chapters 1 through 9 are designed to teach you details of using FlowJo. For this demonstration, you will load a sample data set into a previously designed Workspace that already has gates, statistics, and graphical reports designed. You can think of this workspace as a "Template," which could be used over and over for similar sets of experiments.

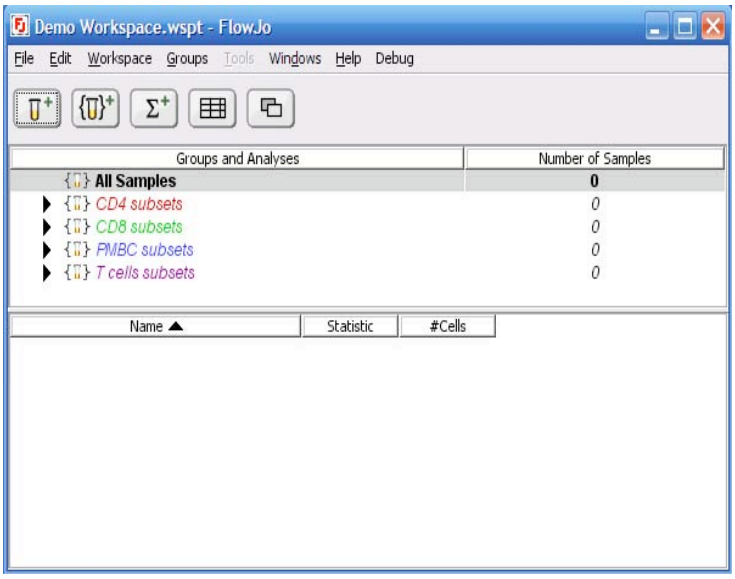
To begin this tutorial, first locate the "**Advanced Tutorial Data and Workspaces**" Folder on your PC (either copied from the CD-ROM that came with FlowJo, or downloaded from the FlowJo web site). Open this folder; you will see a number of Workspace documents (.wsp) and a folder containing the Tutorial data (inside it has 2 folders of experimental data collected on different dates). Locate the workspace document named **Demo_Workspace.wspt** and double-click it to launch FlowJo.

The unlicensed version of FlowJo can only read demonstration data from Tree Star, Inc. You can run this tutorial, or look at special FCS files that we provide, but it will not read data from your lab until you have obtained a serial number or dongle from Tree Star. Serial numbers are machine specific, so you need a serial number for every PC on which you will run the program. We provide free *evaluation* serial numbers that let you run the program for a specific time period (generally thirty days). In this way you can try out FlowJo with your own data files.


FlowJo License Information
About Dongles
FlowJo does not detect a security key(dongle). If you have one attached, please click on "About Dongles" to view our trouble-shooting web page.
Register License
If you have just purchased FlowJo, and do not have a serial number, go to the web to get your serial number.
Purchase License
If you do not own the program, but wish to , you can buy it online.
Request Trial License
Otherwise, you'll probably want to request a free 30 day evaluation license
If you have been issued a serial number, enter it here, and click the button on the right to activate it.
Use Serial Number
SN must be 16 characters
alternately, you may run in Demo Mode, using only the provided data files.
Run in Demo Mode
Hardware Address: 001422E6B836
Quit

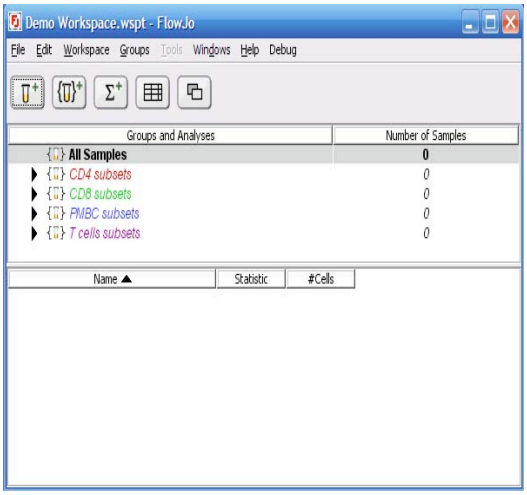
When you first run FlowJo, you will be presented with the dialog window shown on this page. (Once you enter a serial number, FlowJo won't ask for it again). For now, you can click the **Run In Demo Mode** button and continue.

Later, you'll want to request your own serial number to try out the program with your own data files. For more information about registering, point your web browser to www.flowjo.com/try.html, or send an email to FlowJo@treestar.com.



Open the Demo Workspace by double-clicking on PC Workspace.wspt in (see the window at right). The organization of the ***Workspace*** window is more fully described in later chapters. For now, realize that the upper half of the window consists of ***Sample Groups*** which specify analyses (gates and statistics) to be applied to appropriately-stained samples, and the lower half of the window is the list of all samples in the currently selected G***roup*** (The "All Samples" group always includes every sample in the Workspace).

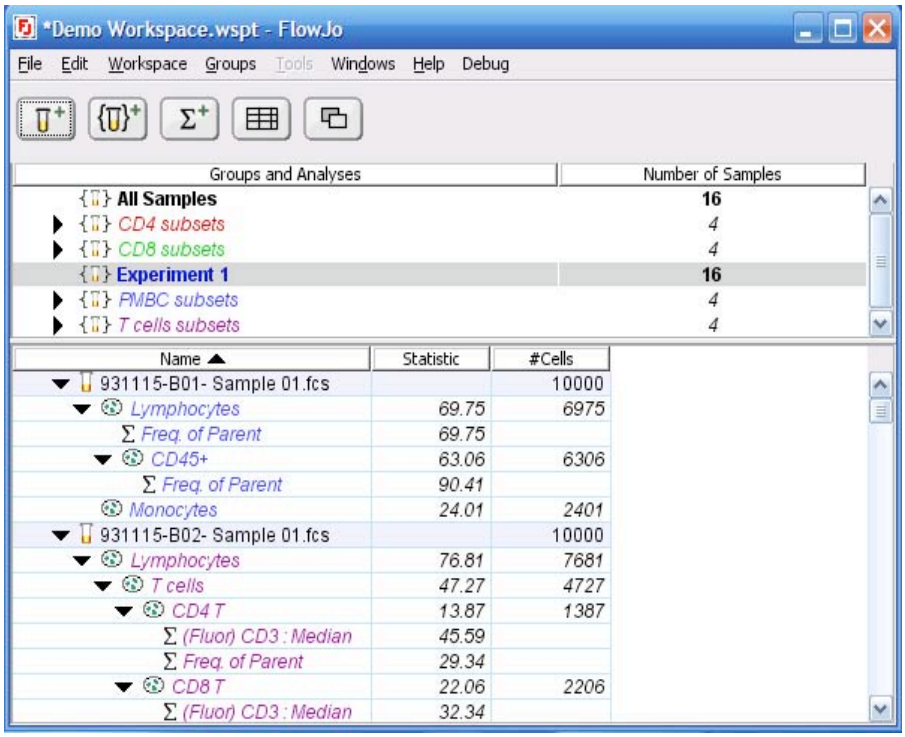
Because this is a template workspace, there are no samples in it. Adding appropriately stained samples will produce the analysis that the template was built to perform.



There are several ways to load data into a workspace; perhaps the easiest is to drag the Folder "Example 1" into the FlowJo window: click on "Experiment 1" in Windows Explorer, and, without releasing the mouse, drag it over the lower half of the ***Workspace*** window.

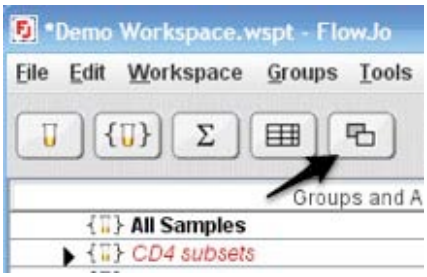
When you release the mouse button, FlowJo examines all of the files in the folder you dragged, and loads all FCS data files it finds. FlowJo can read data files collected on cytometers from any manufacturer. The workspace now reflects the new samples — and analyses (see next page). In this experiment, each sample was named by the date of collection (931115), a code related to the antibody panel (B, C, D, or E), and a sample identifier (e.g., "Sample 01").

Just by adding the data files to this workspace, FlowJo has already accomplished most of the analysis steps for this data. Each file was examined to see what reagent panel was used to stain the cells. Depending on the panel, FlowJo added the data file to one or more Groups. Then, the gates, statistics, or other analyses specified by each group were automatically added to the sample. Thus, gates and statistics specifically designed for each reagent panel were applied only to the sample tubes for which they were designed. You can scroll up and down the sample list to see the variety of different gates and statistics that were applied to each sample.



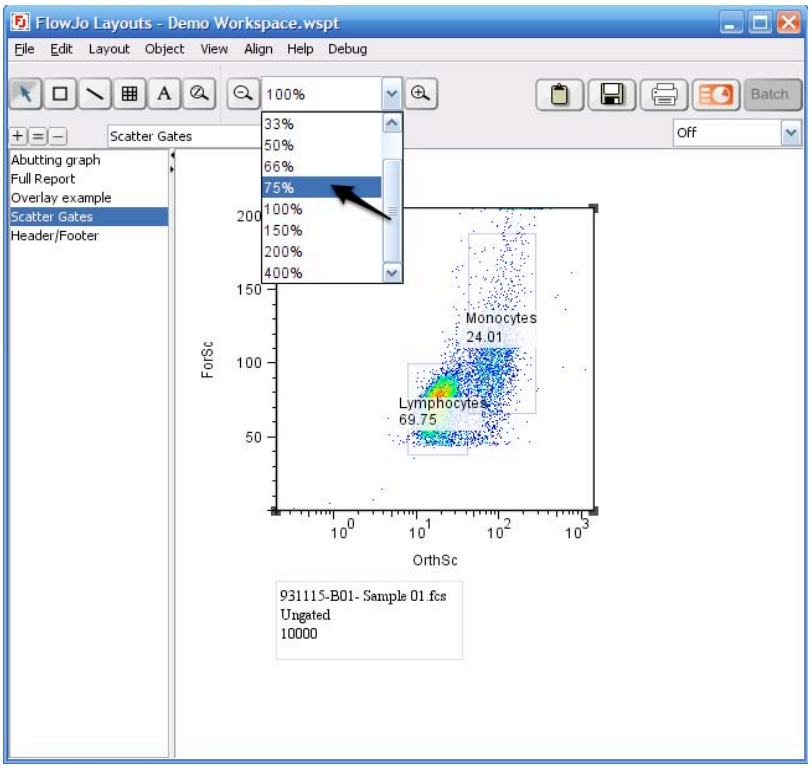
This workspace also has several graphical layout reports saved with it. FlowJo saves not only analyses, gates, and statistics with workspaces, but also any tabular and graphical reports, compensation matrices, kinetics and cell cycle analyses, and calibration standards. Everything you do is recorded and saved so that you don't have to redo the same steps.

To generate one of these graphical reports, you need to open the Layout Editor Window. You can either select this from the Windows menu, or click on the Layout icon in the Workspace window (as shown at right).



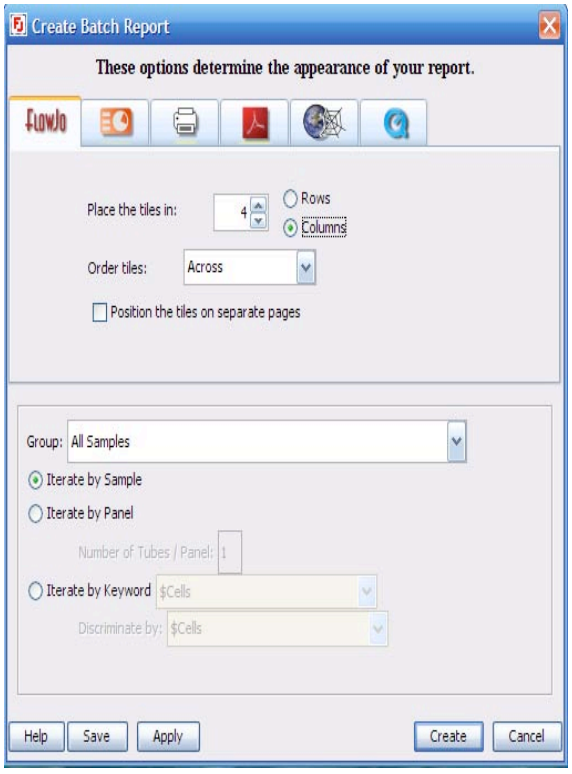
FlowJo now shows you the ***Layout Editor*** window, opening one of the four graphical layouts that have been designed for this workspace (see graphic on the next page). In the left-hand portion of the ***Layout Editor*** window, you will see a list of different layout templates. To fully see the "Abutting Graph" you need to click on the "CD4 subsets" group in the Workspace. For the "Overlay example" choose either the "All Samples" or the "Experiment 1" group and select one of the menu entries showing the Patient ID on the top right near $Cells. Finally, click on the Layout named "Scatter Gates".

One of the important first steps in analyzing an experiment is to make sure that all of the scatter gates are appropriate for each sample. You will use this layout to verify the scatter gates for all 16 tubes collected in this experiment.



Change the magnification to 75% (as shown here). This gives you a better view of the Forward vs. Side scatter dot plot. If you wanted, you could now cycle through every plot by turning iteration on and choosing "Sample" from the drop down list and clicking successively on the "Next" or "Previous" sample arrows (just below the ***Batch*** button). However, you can use FlowJo's Batch analysis to automate this process. Click on the **Batch…** Button in the upper right.

From here you can choose from a variety of report formats. Next to **Place the tiles in:** type a 4 in the text box. Choose "**columns**". Click **Create** to view the same gates applied to each of the samples in the *Workspace*.



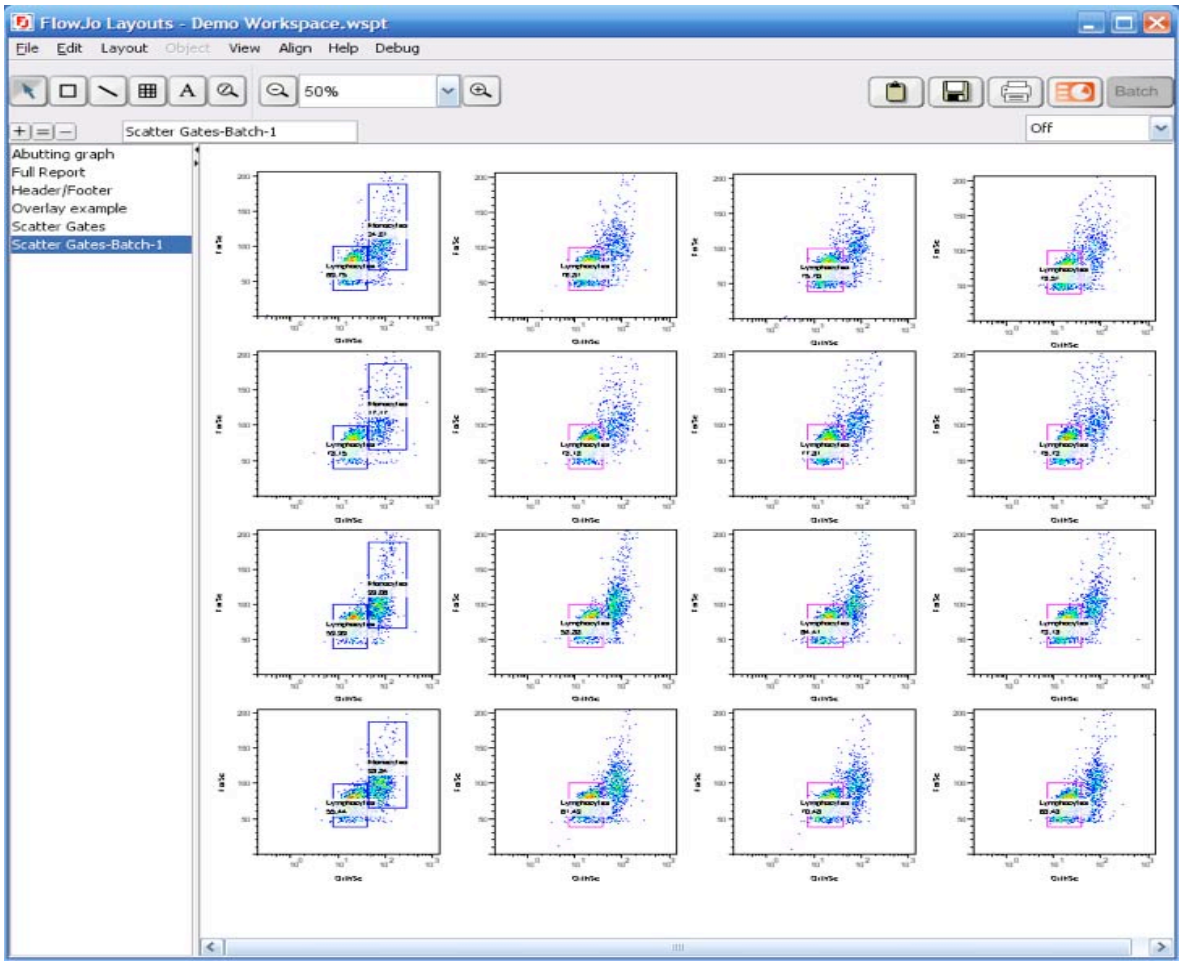
Then try out the different Batch button options:

- **Direct to printer** to print out the output,
- **Power Point Data** to save the output in PowerPoint slide format,
- **Movie** to play each successive sample in frames of a QuickTime animation,
- **Web Report** to view the output in a browser, or
- **PDF** to generate a report In the PDF file format.

In Chapters 8 and 9, you will learn how to arrange the graphics in the Tiles view to be printed in exactly the format you desire.

To demonstrate how fast this whole process can be, there is a second experiment's data also supplied with the Tutorial (in the folder Experiment 2). This is data for 14 more individuals (and is therefore much more extensive than Experiment 1). Drag this folder onto the Workspace (like you did for Experiment 1), and create the batch outputs as before.

With a well-designed ***Workspace***, you can completely analyze and generate custom graphics (and tabular) reports in a matter of just minutes for an entire experiment.



This is the true power of FlowJo: Experiment-Based Analysis.

Lesson 1: Workspaces and Basic Data Display

FlowJo provides an integrated environment, the Workspace, for the viewing and analysis of flow cytometric data. The Workspace contains a list of all of the samples that you load into it, the gates that you apply, the statistics that you calculate, and the table templates and graphical layout templates that you design.

Think of the Workspace as your experimental notebook. In general, you will create a different Workspace for each kind of experiment that you perform. A Workspace may contain multiple samples collected on various days – and can provide a rigorous way to organize your data analyses.

To begin analyzing data in FlowJo, you will need to create a new Workspace and add data files into it. Later, you can simply double-click on this Workspace to continue work. For this tutorial, you will create a new Workspace and load a set of data files from a single collection. You will learn how to carry out basic analyses, including batch analyses. At the end of the tutorial, you will load a second experiment with a similar set of samples, collected on a different day. You will use all of the batch analysis tools you created in the first set of analyses and quickly derive detailed statistical and graphical information (in the forms of tables and printed graphical layouts) for both sets of samples.

Workspaces are the basic environment that FlowJo uses to help you organize your data. They don't actually contain the raw data. Instead, Workspaces point to the data that you load. If you move the raw data files to another disk, FlowJo will ask you to find the data the next time it needs that information. Workspaces do store much of the information about samples, as well as all of the analyses (gates and statistics) that you have previously computed.

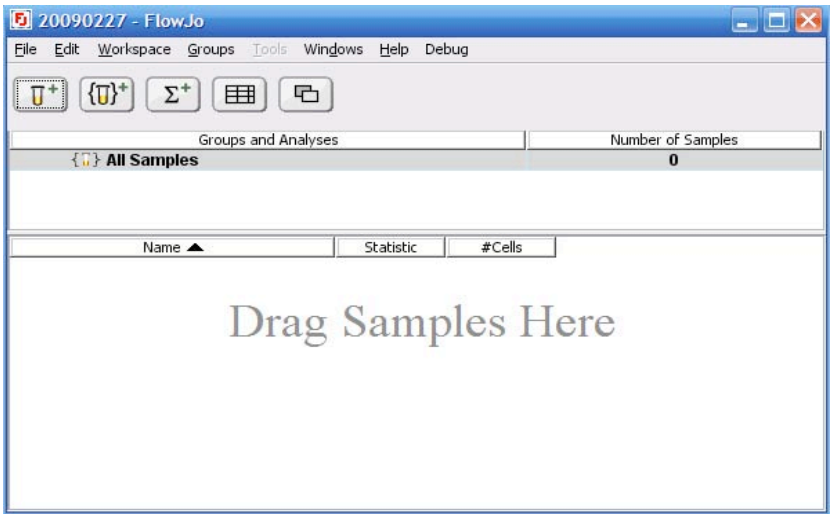
Introduction to the Sample Data Used in this Tutorial

There were two experiments, each performed on separate days. The experiments involved 3-color immunophenotyping of PBMC preparations from human adults. Each preparation was split into 4 aliquots to be stained with four different combinations of antibodies (see table); thus, there are a total of four tubes per PBMC preparation. (In general, we refer to each tube, or data collection, as a sample. In this experiment, there are four samples for each cell preparation).

In this experiment there are four patients; in the second (folder "Experiment 2"), there are 14. The stain combinations used are as follows:

Stain #	FITC	PE	Cy5PE
1	CD14	CD16	CD45
2	CD3	CD8	CD4
7	CD62L	CD45RA	CD4
8	CD62L	CD45RA	CD8

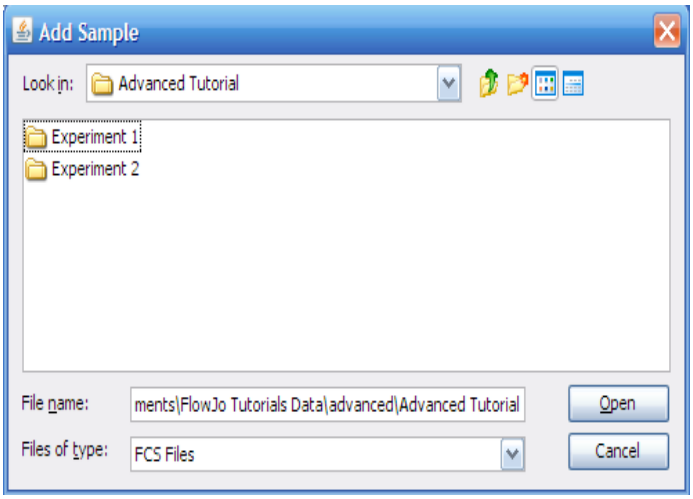
As you work through this tutorial, the goal is to analyze the frequencies of some of the subsets that can be identified using these antibody stains, and to collate graphs and statistical information about subsets.



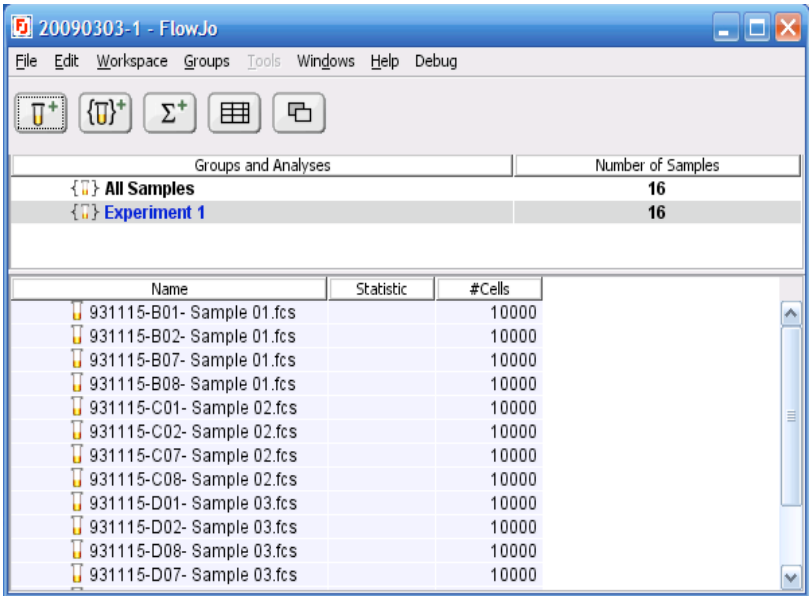
Once you have launched FlowJo, you will be shown a window similar to that shown to the right. Note the ***Help*** Menu near the top right. Most windows in FlowJo have a ***Help*** menu or a ***Help*** button. When you click on ***Help***, FlowJo launches your standard web browser to get the on-line version of Help (or, you can press the ***F1*** key on your keyboard at any time). The help for FlowJo is HTML-based; when you ask for help, you will automatically be shown information about the active window. Within the browser, you can navigate to all of the help pages for FlowJo, and learn how to operate the program. You can go directly to the main FlowJo help page by selecting ***Help > FlowJo Reference Manual*** in the ***Workspace*** window.

The ***Workspace*** window is divided into three parts. The top part is a button bar. The button bar has controls to let you add components to the Workspace, like data files, statistical analyses, etc. As you move the mouse over a control button, FlowJo displays a description of that control's action in a tool tip. The second part of the Workspace (above the central divider) is a list of sample groups. Later, you will see how to group sets of samples together for batch analysis. The bottom part of the window includes a list of the samples in the Workspace.

Now, click on the left-most button, Add Samples... You will see a standard Java File dialog; navigate to the **Advanced Tutorial** folder that you downloaded with the tutorial (see right).



Highlight the folder **Experiment 1**, and click on the **Open** button at the bottom to add all of the files in this folder (16 samples, one for each staining tube: four sets of PBMC, with four stains each). Your ***Workspace*** window should look as shown.

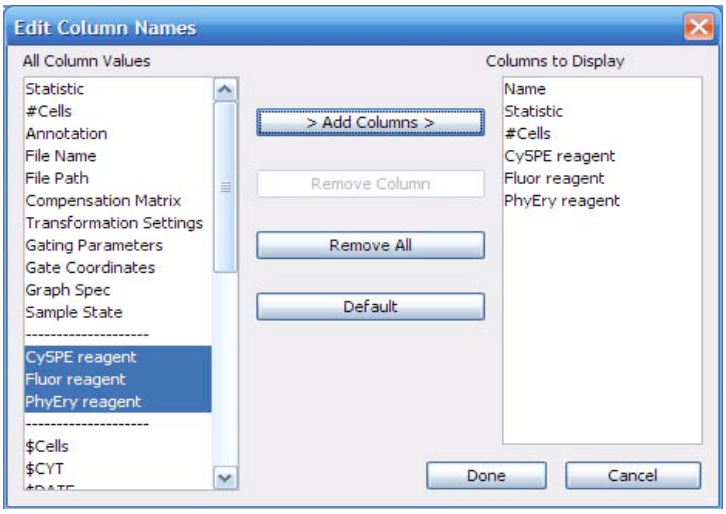


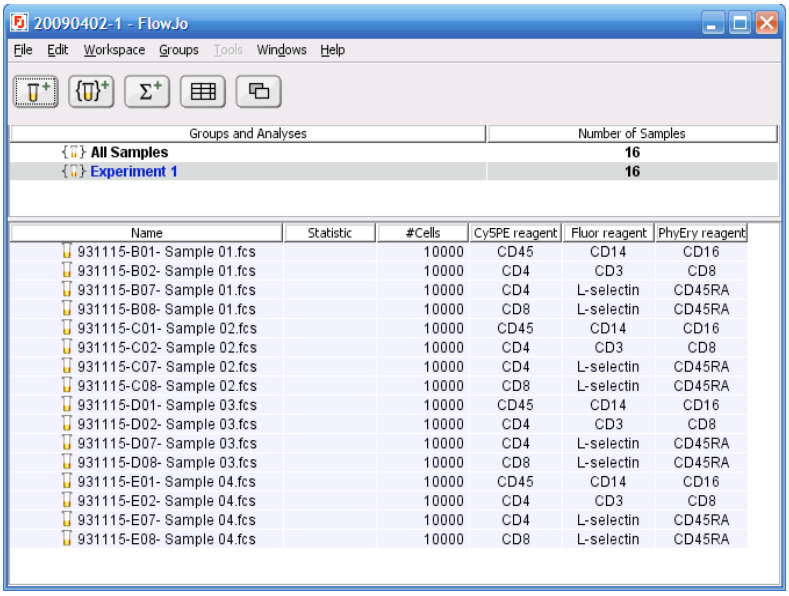
Two things have happened: FlowJo created a new sample group with the same name as the folder containing the FCS files (**Experiment 1**), and the samples are now listed in the bottom portion of the window. (The numbers to the right of each group show how many data files are included in that group). You can resize the ***Workspace*** window as with any PC window; you can resize the group and sample portions by clicking and dragging on the dividing bar between them.

Each file is shown as a test tube icon followed by the title of the sample's file. To the right is shown the number of events that were collected in that file.

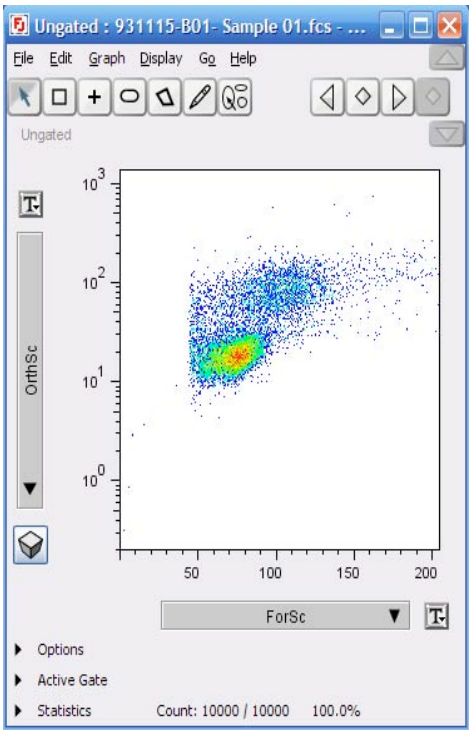
You can add additional columns to the Workspace display that show the stains used for each sample, or any other keyword value found in the FCS data file. You may also sort the sample list on the basis of any of these values by double clicking the column name.

To add the reagent list to the workspace view, click on and select the command ***Edit Columns...***. FlowJo shows the window on the right. Move the desired column headers from the list of available attributes (on the left) to the list of visible attributes (on the right). Click **Add Columns** to display the settings. Click **Done** to accept the changes, and the Workspace view will add the additional columns to the table.



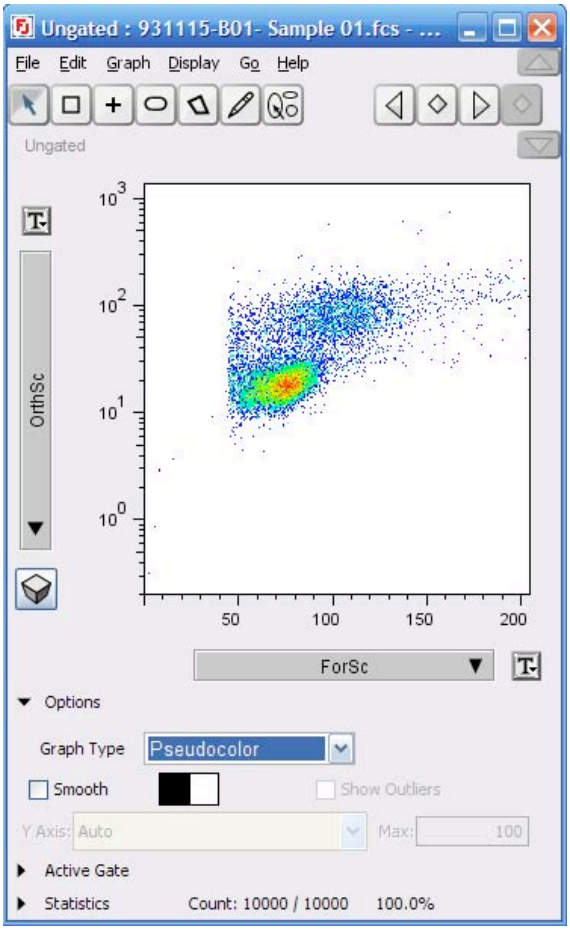

20090402-1 - FlowJo
File Edit Workspace Groups Tools Windows Help
Groups and Analyses
Number of Samples
All Samples 16
Experiment 1 16
Name Statistic #Cells CySPE reagent Fluor reagent PhyEry reagent
931115-B01- Sample 01.fcs 10000 CD45 CD14 CD16
931115-B02- Sample 01.fcs 10000 CD4 CD3 CD8
931115-B07- Sample 01.fcs 10000 CD4 L-selectin CD45RA
931115-B08- Sample 01.fcs 10000 CD8 L-selectin CD45RA
931115-C01- Sample 02.fcs 10000 CD45 CD14 CD16
931115-C02- Sample 02.fcs 10000 CD4 CD3 CD8
931115-C07- Sample 02.fcs 10000 CD4 L-selectin CD45RA
931115-C08- Sample 02.fcs 10000 CD8 L-selectin CD45RA
931115-D01- Sample 03.fcs 10000 CD45 CD14 CD16
931115-D02- Sample 03.fcs 10000 CD4 CD3 CD8
931115-D07- Sample 03.fcs 10000 CD4 L-selectin CD45RA
931115-D08- Sample 03.fcs 10000 CD8 L-selectin CD45RA
931115-E01- Sample 04.fcs 10000 CD45 CD14 CD16
931115-E02- Sample 04.fcs 10000 CD4 CD3 CD8
931115-E07- Sample 04.fcs 10000 CD4 L-selectin CD45RA
931115-E08- Sample 04.fcs 10000 CD8 L-selectin CD45RA

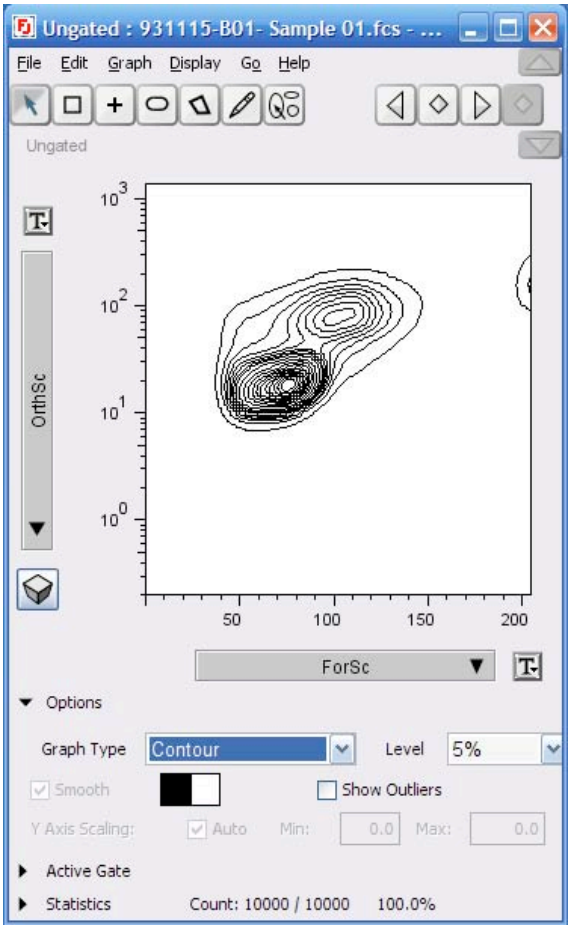
To view the data for any sample, double click on one of the lines in the sample list. When you double-click on the first line in the sample list, you will see the graph shown below.



This is a ***Graph*** Window. A ***Graph*** window will show you a plot of the data. There are several different kinds of plots that can be used to display the data. The default plot that you see first is determined by the Preferences setting. To change the Graph, click on the **Options** disclosure triangle at the bottom of the ***Graph*** window.

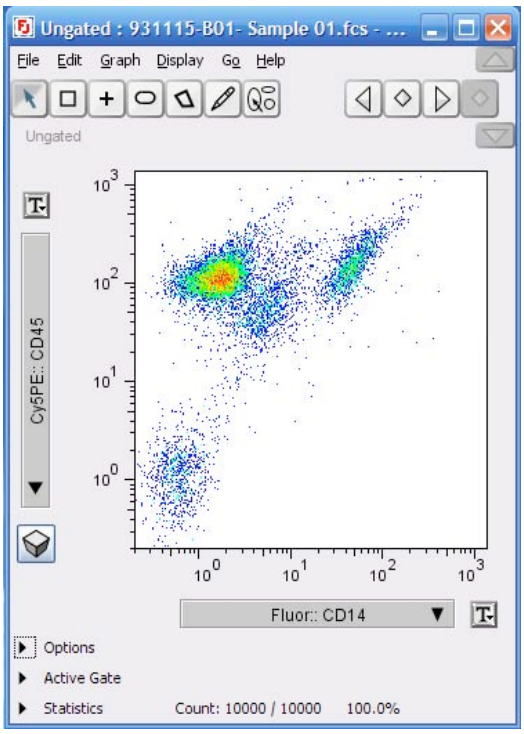
This button brings up the options shown to the right. By clicking on the graph type drop-down menu you can select among different types of graph plots. Select contour to see the graph displayed below.

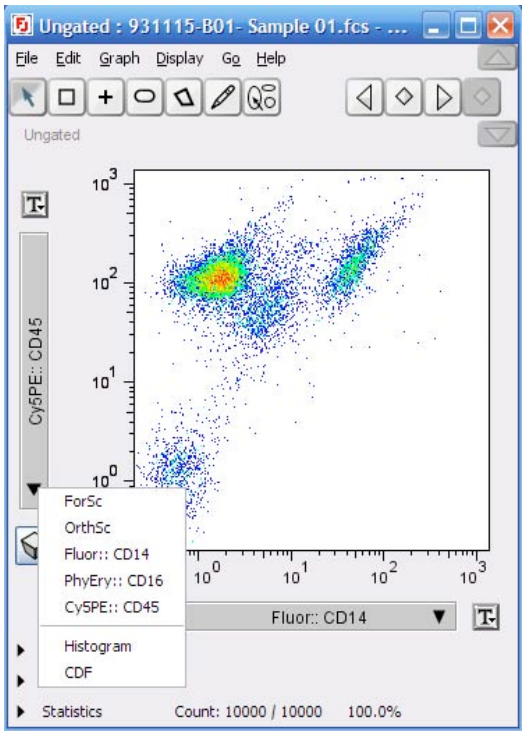




If you click on the menu ***Edit > Save State to Preferences,*** this plot will become the default type of plot to be opened. Note that this selection only applies to workspaces you create subsequently; it doesn't affect the currently open graphs. Almost every window in FlowJo has the ***Save State to Preferences*** command that allows saving the options currently displayed in this window as the default.

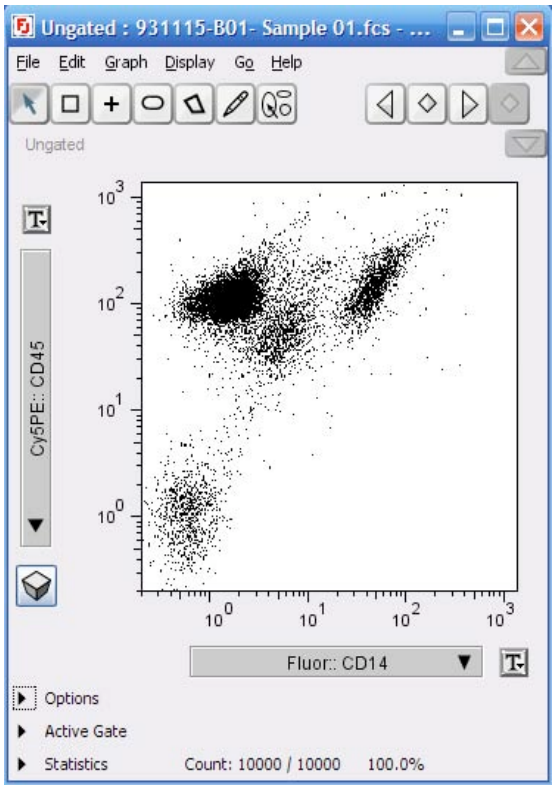
Again select the pseudocolor plot type. To change the graph's parameters click on the axis labels and select the parameter you wish to view from the menu display. Change the X axis to "CD14" by clicking on the X axis and selecting Fluor::CD14. After changing the Y axis to "CD45" the data graph will look like the one on the next page.



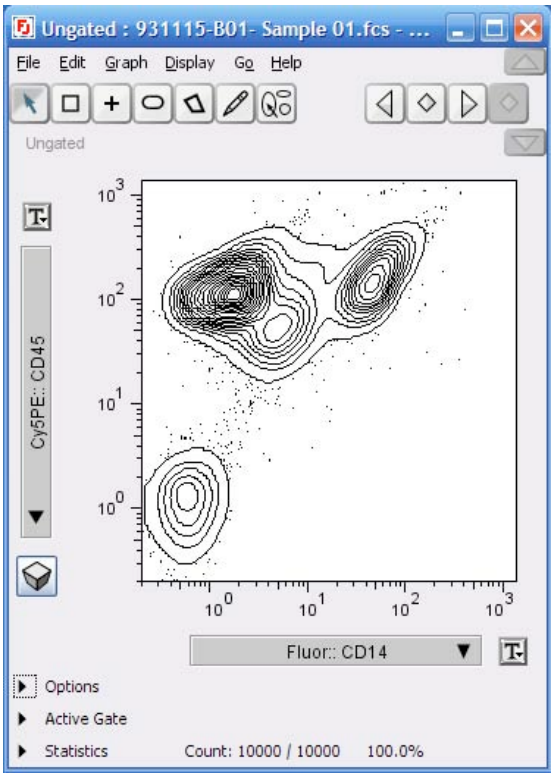


You may also choose "Histogram" or "CDF" (cumulative distribution function) to create a univariate plot. Experiment with different graph types and different options. You can also change individual options by selecting them from the ***Display*** menu at the top of the graph window**.** (Try selecting ***Use Large Dots*** under the Display tab for the pseudocolor plot; this type of plot – low resolution pseudocolor – may be the best for slide presentations). In general, the probability contour plots give the best representation of the data, though they are lacking in that they do not show rare events. To work around this, FlowJo provides the option of displaying "Outliers." Outlier plots combine the density estimation information of the contours with the rare event information of a dot plot. The density plots (either grayscale or pseudocolor) are superior to dot plots in that they provide density information (i.e. the number of events within an area) by using different colors or shades. Grayscale plots may be useful for publication and pseudo-color plots for slide presentations. Shown on the next page are examples of some of the other types of plots supported by FlowJo.

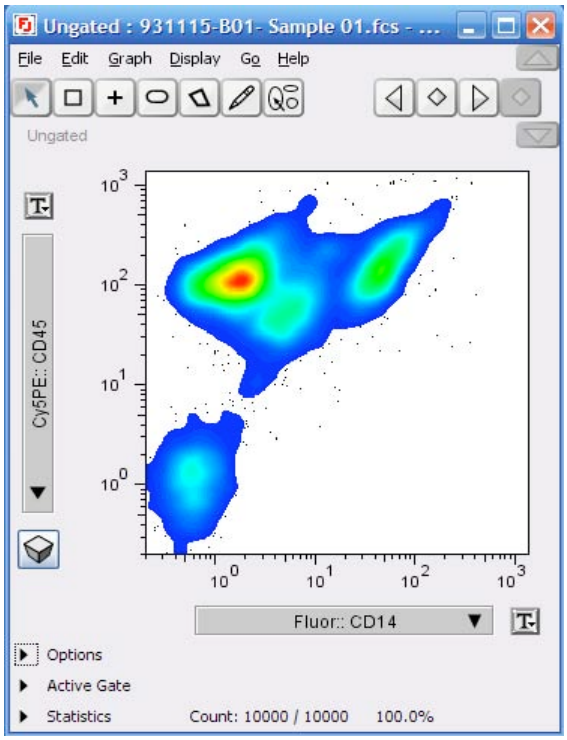
When you close a ***Graph*** window, FlowJo records the plot style and axes. When you next open the graph for that subset, you will see the graph as it was when you closed it. This is, in part, how FlowJo saves the environment across analysis sessions.



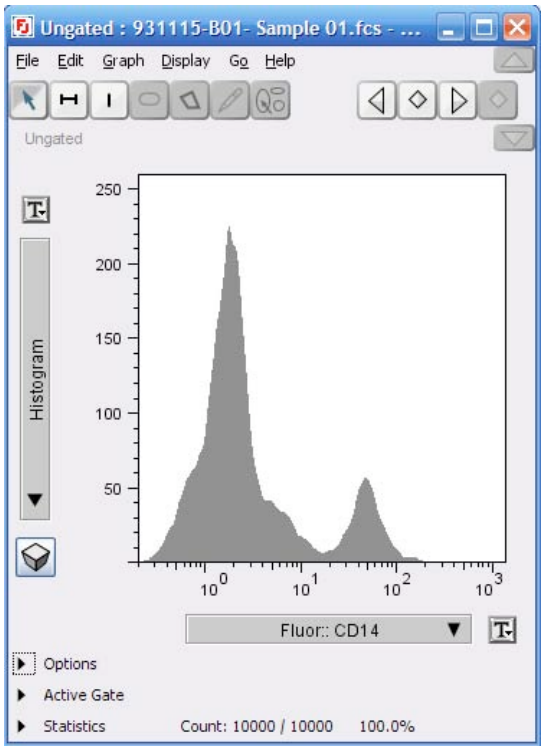
Dot plot



Contour plot with outliers



Smoothed pseudocolor plot

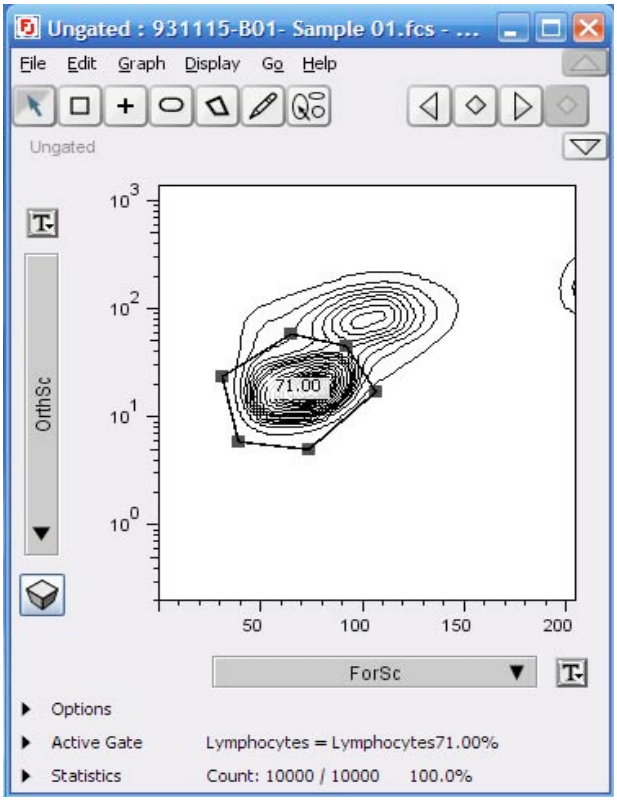


Histogram plot

Lesson 2: Gating and Statistics

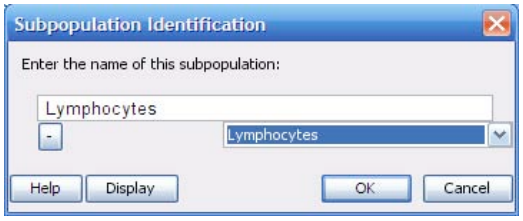
In this lesson, you will learn how to gate data to create subsets. You will also learn how to calculate a variety of statistics from the data. Subsets in FlowJo are exactly like subsets in biology: they representing a portion of the entire collection with specified properties (e.g., Lymphocytes are those cells with low forward and side scatter). You will always be asked to name a subset; the name you choose is important for organization within FlowJo. Select a name that is meaningful to you, as this will help you keep track of your analyses.

Creating a gate is simple: Choose the polygon gate icon (fifth from the left) – then just click inside a graph (the mouse will appear as a cross-hair) and move the mouse. Each time you want to change directions, click. This is the same process you would use to draw a polygon in any drawing program. If you hold down the ***shift*** key, you get horizontal, vertical or diagonal lines; you can use the ***delete*** key to remove a polygon while drawing it. Either click on the first point or double-click at any time to close the polygon and create the gate.



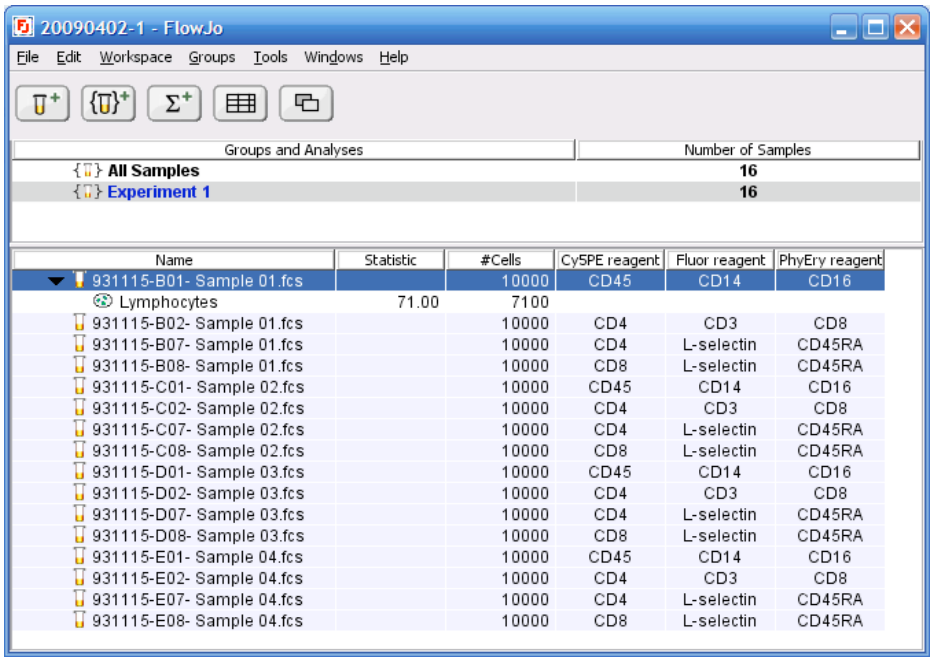
Open the first file in the sample Workspace you created and change the graph to Forward vs. Orthagonal-scatter and the graph type to contour. You will now create a lymphocyte gate: with the polygon tool draw a new gate around the lower population, as shown on the right.

As soon as you close the polygon, FlowJo asks you to name the subset that you have just gated (see below) FlowJo provides a default name; *however, you should choose a name that describes the population that you gated.* Names are very important within FlowJo analyses. The success of your future analyses depends on having analyses with descriptive names. For example, in this case you have gated lymphocytes, so name this subset "Lymphocytes". (The Help button will provide more information about naming subsets). Type the name into the box or choose from the pull down menu. You can add names you type to a list of favorites by clicking on the plus icon. If you click on the **Display** button, FlowJo then creates the gate, and opens a new ***Graph*** window showing only the events contained within the gate you just drew. For now, just click on the **OK** button.

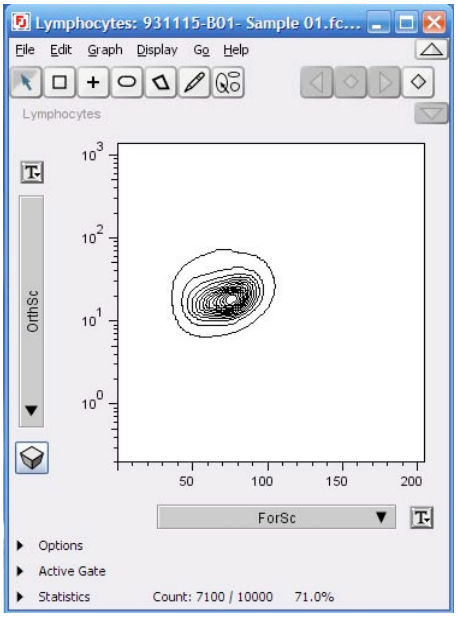


You will note some changes to the ***Graph*** window. The active gate line (second line from the bottom) has the name of the currently-selected gate and the fraction of events in the entire sample that fall into the subset.

If you double click on the graph inside the Lymphocytes gate, FlowJo opens a new graph showing the data contained only within the Lymphocytes gate. Also, there is a new entry in the ***Workspace*** window (see example, below).

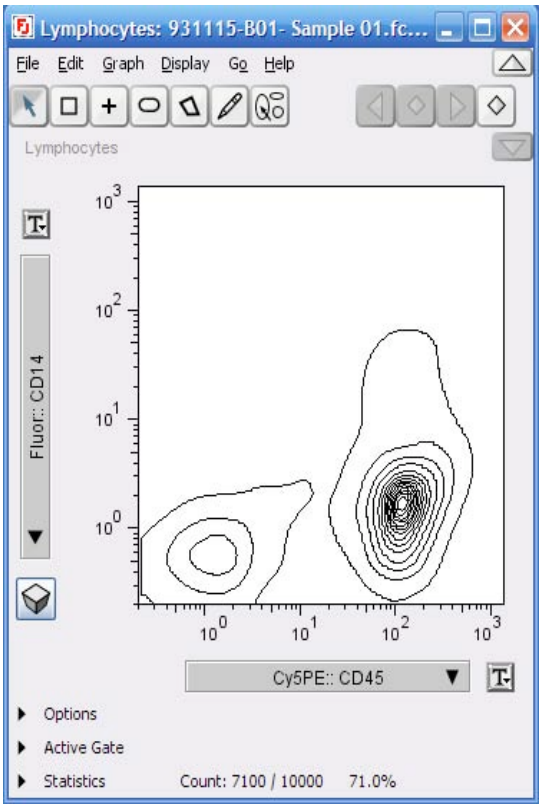

20090402-1 - FlowJo
File Edit Workspace Groups Tools Windows Help
Groups and Analyses
Number of Samples
All Samples 16
Experiment 1 16
Name Statistic #Cells CySPE reagent Fluor reagent PhyEry reagent
931115-B01- Sample 01.fcs 10000 CD45 CD14 CD16
Lymphocytes 71.00 7100
931115-B02- Sample 01.fcs 10000 CD4 CD3 CD8
931115-B07- Sample 01.fcs 10000 CD4 L-selectin CD45RA
931115-B08- Sample 01.fcs 10000 CD8 L-selectin CD45RA
931115-C01- Sample 02.fcs 10000 CD45 CD14 CD16
931115-C02- Sample 02.fcs 10000 CD4 CD3 CD8
931115-C07- Sample 02.fcs 10000 CD4 L-selectin CD45RA
931115-C08- Sample 02.fcs 10000 CD8 L-selectin CD45RA
931115-D01- Sample 03.fcs 10000 CD45 CD14 CD16
931115-D02- Sample 03.fcs 10000 CD4 CD3 CD8
931115-D07- Sample 03.fcs 10000 CD4 L-selectin CD45RA
931115-D08- Sample 03.fcs 10000 CD8 L-selectin CD45RA
931115-E01- Sample 04.fcs 10000 CD45 CD14 CD16
931115-E02- Sample 04.fcs 10000 CD4 CD3 CD8
931115-E07- Sample 04.fcs 10000 CD4 L-selectin CD45RA
931115-E08- Sample 04.fcs 10000 CD8 L-selectin CD45RA

Underneath the sample you just gated, indented one level, is a new row. This new row represents the subpopulation defined by the gate. This row begins with a subset icon followed by the name that you gave to the subset. To the right, you will find the frequency of these events (within the sample) and the total number of events in this gate. Any operation that can be performed on a sample can also be done to a subpopulation. You can double-click on the **Lymphocytes** subset to open its graph, gate within that subset, etc. Double-click on this population. You will see the graph at right.

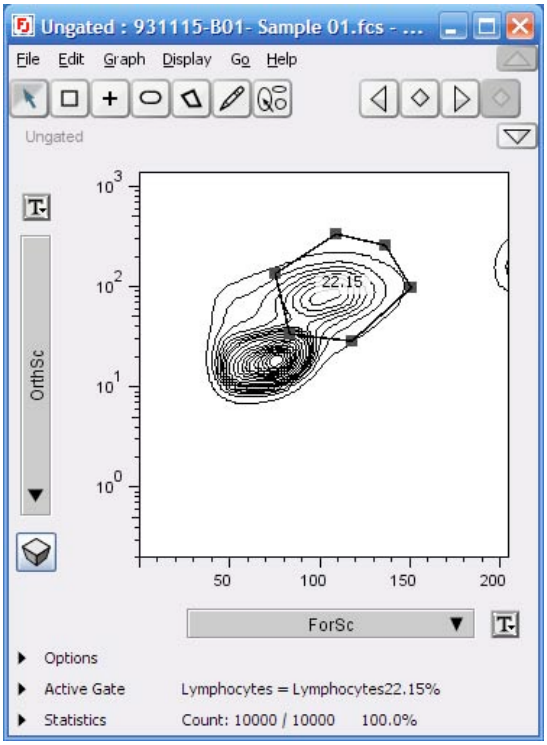


This is the contour plot for the events falling within the lymphocyte gate. Note that the number of events in this graph (**Count** at the bottom of the window) is 7100. This is the number of cells falling within the lymphocyte gate. You will also see that the Up-arrow button is now active. Clicking on this button opens the graph you used to create this subset (i.e., you will see the gate for this subset). The Up-arrow can be used to navigate to the parent population; the Down-arrow navigates to the subset. Click on the down-arrow (or double-click on the gate in the graph) to show a new ***Graph*** window for the subset.

Change this graph to CD14 vs. CD45; your graph now will look like the one to the right. Note that most of the events are CD45 positive and CD14 negative: this is exactly what we expect for lymphocytes in PBMC. The CD45 negative population is probably red blood cells that contaminated the cell preparation. Move this window to the side, and click on the Up-arrow button to make the parent population the active window. You should be able to see both graphs simultaneously.



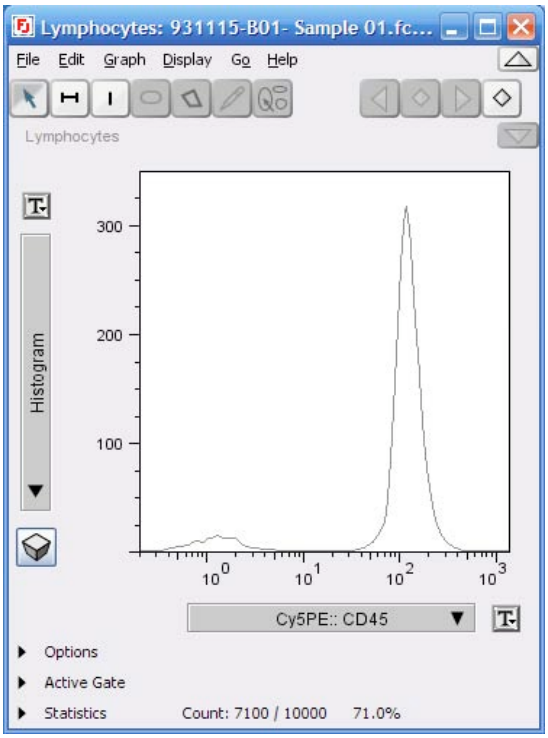
FlowJo uses dynamic recalculation of gates. This means that whenever you adjust a gate, FlowJo automatically updates all visible windows to reflect that change. Move the mouse over the lymphocyte gate in the ***Graph*** window; the cursor changes to a cross. If you now click and drag, you will move the gate. Move the gate so that it is over the monocyte population as shown below.



FlowJo instantly updates the graph of the subset; you will be able to see these dynamic changes in the graph window.

The new gate includes events that are all CD45-positive, and a large fraction are also CD14-positive (i.e., monocytes). You can continue to move the gate around, and see exactly how the gating affects the subpopulations. These changes also occur when you make minor modifications to a gate (like clicking and dragging a single vertex).

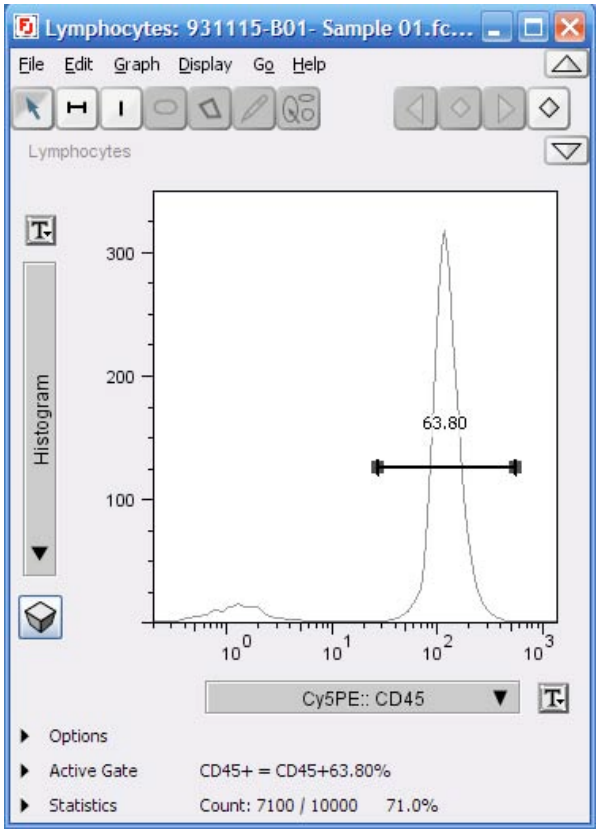
Move the gate back to the lymphocyte population so that the gate accurately reflects lymphocyte cells. Change the graph for the lymphocyte population – double click on the gate in the graph window to get to the Lymphocyte ***Graph***, or simply select the corresponding ***Graph*** window – so that it displays a histogram of CD45. Select Histogram using the Y axis pop-up (or use the ***Options*** disclosure triangle to define the graph type).



The graph should now appear as shown on the right.

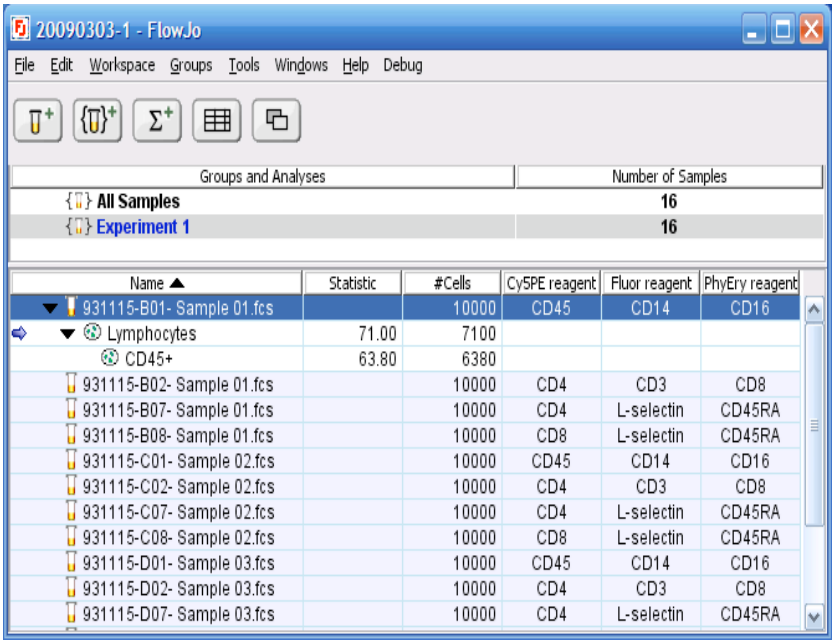
If you wish, you can change the Y axis scaling on univariate plots through the controls in the options opened by the options disclosure triangle.

Creating a gate on a histogram is the same as creating a gate on a bivariate plot: click and drag in either direction. Make a gate to include only the CD45-positive cells:



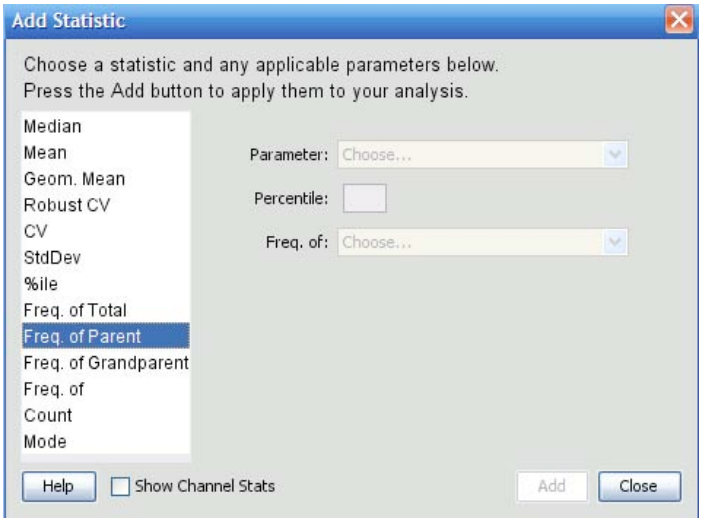
Select the range gate tool and click on one side of the peak and drag to the other side. When you let go of the mouse, FlowJo again asks you the name for this new subpopulation (subset of cells). Type in "CD45+" as the name of the subset.

The ***Graph*** window now displays the gate and the statistics for that gate, as shown left. The vertical placement of the gate is irrelevant; you can drag the gate horizontally or vertically by clicking on the horizontal bar (the cursor becomes a hand) and moving it around. You can change the upper or lower boundaries by clicking on one of the handles and moving it.
The ***Workspace*** window now has a new entry, as shown in the example on the next page.


20090303-1 - FlowJo
File Edit Workspace Groups Tools Windows Help Debug
Groups and Analyses
Number of Samples
All Samples 16
Experiment 1 16
Name Statistic #Cells Cy5PE reagent Fluor reagent PhyEry reagent
931115-B01- Sample 01.fcs 10000 CD45 CD14 CD16
Lymphocytes 71.00 7100
CD45+ 63.80 6380
931115-B02- Sample 01.fcs 10000 CD4 CD3 CD8
931115-B07- Sample 01.fcs 10000 CD4 L-selectin CD45RA
931115-B08- Sample 01.fcs 10000 CD8 L-selectin CD45RA
931115-C01- Sample 02.fcs 10000 CD45 CD14 CD16
931115-C02- Sample 02.fcs 10000 CD4 CD3 CD8
931115-C07- Sample 02.fcs 10000 CD4 L-selectin CD45RA
931115-C08- Sample 02.fcs 10000 CD8 L-selectin CD45RA
931115-D01- Sample 03.fcs 10000 CD45 CD14 CD16
931115-D02- Sample 03.fcs 10000 CD4 CD3 CD8
931115-D07- Sample 03.fcs 10000 CD4 L-selectin CD45RA

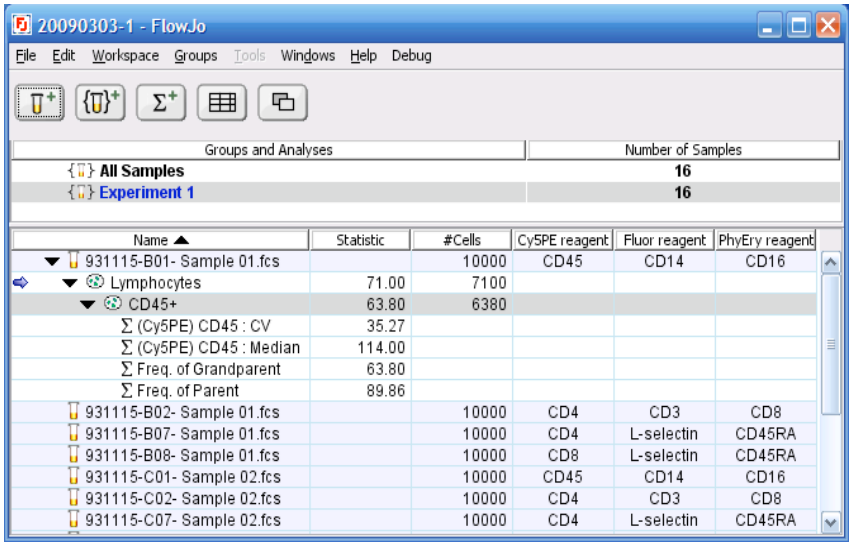
Note that the new entry is below Lymphocytes and is further indented. This is because the new population is a subset of Lymphocytes. The Workspace reflects this hierarchy. The numbers to the right indicate the fraction of events falling within the gates. Thus, 71.0% of the sample's events fall in the Lymphocyte gate; 63.8% of the Lymphocytes are CD45-positive.

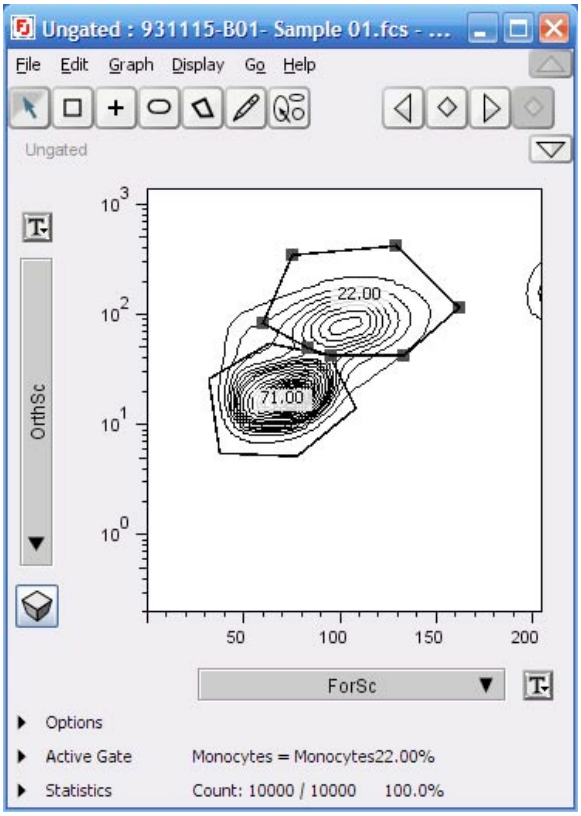
Now we will add some statistics to one of these subsets. In the ***Workspace*** click on the CD45+ row so that it is highlighted, and then click the middle button in the button bar (the Sigma button). This indicates that you want to calculate a statistic on the currently selected subset. You will now see the ***Statistics*** window, as shown to the right.



On the left side of the ***Statistics*** window is a list of the statistics available. Some of these statistics require that you choose a parameter on which to calculate the statistic (for example, Mean Fluorescence). If you wish to compute a specific percentile of one of the parameters, you will type the percentile into the numerical box. For now, click on **Freq. of Parent**, and then click on **Add.** Also, add the **Freq. of Grandparent**, and add the **Median** and **CV** for Cy5PE (select the parameter from the **Parameter** popup menu). If you click on **Help**, a corresponding web page will be opened with your web browser that gives you complete information about the various statistics. When you have finished adding statistics to this population, click the **Close** button to close the window. Once you close the ***Statistics*** window, your ***Workspace*** window will have several new entries as shown on the next page.

The new lines begin with a statistics icon. (Click in the column name bar, on the vertical dividers. Drag to show the entire column names). The statistics icon is followed by the parameter on which the statistic is computed (if applicable), the name of the statistic, and the computed value. The **Median** Cy5PE CD45 fluorescence of the CD45+ Lymphocytes is 114.00; the distribution of CD45 has **a Coefficient of Variation** of 35.27%. The **Freq. of Parent** statistic tells you the frequency of CD45+ events within the parent subset (Lymphocytes). You will note that the value 89.86% is equal to 63.80% (the frequency of this subset within the sample) divided by 71.0% (the frequency of lymphocytes within the sample). The **Freq. of Grandparent** shows you the frequency of this subset within the population two levels up – in this case, the sample itself. Thus, for this subset, the Freq. of Grandparent is the same as the frequency of this subset within the sample. These statistics are listed below the CD45+ subset to denote that they are statistics for that subset only.

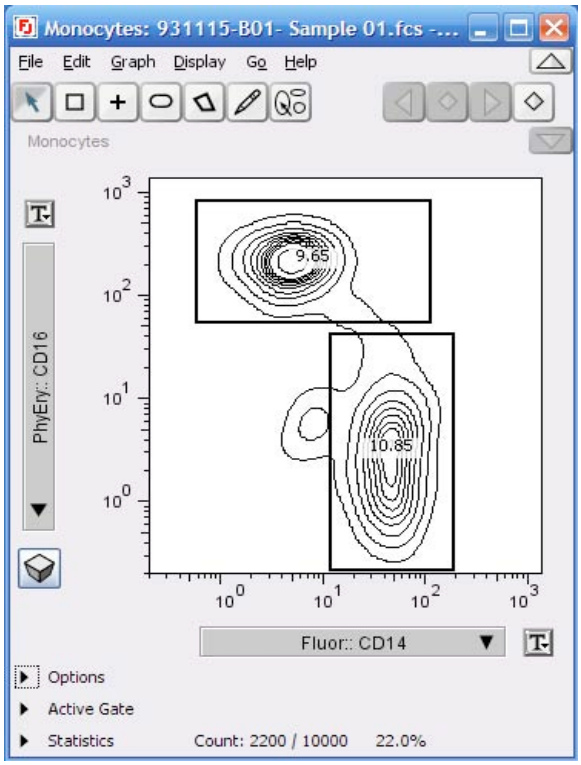

20090303-1 - FlowJo
File Edit Workspace Groups Tools Windows Help Debug
Groups and Analyses
Number of Samples
All Samples 16
Experiment 1 16
Name Statistic #Cells Cy5PE reagent Fluor reagent PhyEry reagent
931115-B01- Sample 01.fcs 10000 CD45 CD14 CD16
Lymphocytes 71.00 7100
CD45+ 63.80 6380
Σ (Cy5PE) CD45 : CV 35.27
Σ (Cy5PE) CD45 : Median 114.00
Σ Freq. of Grandparent 63.80
Σ Freq. of Parent 89.86
931115-B02- Sample 01.fcs 10000 CD4 CD3 CD8
931115-B07- Sample 01.fcs 10000 CD4 L-selectin CD45RA
931115-B08- Sample 01.fcs 10000 CD8 L-selectin CD45RA
931115-C01- Sample 02.fcs 10000 CD45 CD14 CD16
931115-C02- Sample 02.fcs 10000 CD4 CD3 CD8
931115-C07- Sample 02.fcs 10000 CD4 L-selectin CD45RA



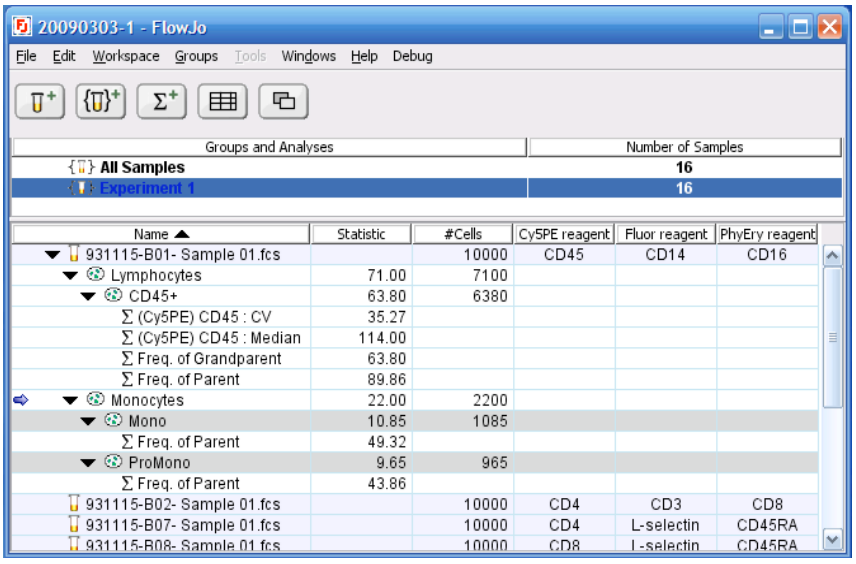
Next, you will create another gate on the sample, this time for monocytes. Double click in the Workspace to open the graph for the first sample, choosing the Forward vs. Orthagonal scatter display option. Draw a gate around the upper population as shown to the left, and name this subset Monocytes.

Note that the currently-selected gate is the one with the square black handles drawn at each vertex. The frequency of events in the currently-selected gate (within the entire sample) is shown at the bottom of the window; when you click on the Down-arrow (or double click on the gate), the ***Graph*** window for the subset will be shown. Click on the Down-arrow to view the monocyte subpopulation. Change the resulting graph to look at CD16 vs. CD14.

Create two gates for the major populations that you see. This time, use the rectangular gate tool to create the gates. Rectangular gates will be computed much more quickly, but this is only evident when you work with very large files (for instance, more than 100,000 events). You may find rectangular gates easier to create. Create two rectangular gates like the graph below.



Name the upper gate (CD16+, CD14-dim) **ProMono** (since this may well be granulocytes; you could name it **Granulocytes** if you wish). The lower right population is principally mature monocytes; name it **Mono**. Look at the Workspace window. You will see the new subsets displayed; click on the ProMono subset and add the **Freq. of Parent** statistic; then do the same for the Mono subset. The Workspace now looks like the Workspace shown below.


20090303-1 - FlowJo
File Edit Workspace Groups Tools Windows Help Debug
Groups and Analyses
Number of Samples
All Samples 16
Experiment 1 16
Name Statistic #Cells Cy5PE reagent Fluor reagent PhyEry reagent
931115-B01- Sample 01.fcs 10000 CD45 CD14 CD16
Lymphocytes 71.00 7100
CD45+ 63.80 6380
Σ (Cy5PE) CD45 : CV 35.27
Σ (Cy5PE) CD45 : Median 114.00
Σ Freq. of Grandparent 63.80
Σ Freq. of Parent 89.86
Monocytes 22.00 2200
Mono 10.85 1085
Σ Freq. of Parent 49.32
ProMono 9.65 965
Σ Freq. of Parent 43.86
931115-B02- Sample 01.fcs 10000 CD4 CD3 CD8
931115-B07- Sample 01.fcs 10000 CD4 L-selectin CD45RA
931115-B08- Sample 01.fcs 10000 CD8 L-selectin CD45RA

Note that the Monocyte subset is indented one level, as is the Lymphocyte subset. Both populations are subsets of the sample. The Mono and ProMono subsets are indented to a second level and are shown below Monocytes, because they are subsets of monocytes. This hierarchy of this gating is visible from the ***Workspace*** window.

This hierarchy is important in that it affects how FlowJo works. It is analogous to a genealogical tree, in that each subset is like a child of its parent population. Thus, in this example, Lymphocytes is a child of the sample, and a parent of CD45+, and a sibling of Monocytes. FlowJo does not allow you to name siblings (i.e., gates on the same population) with the same name – this way no confusion can arise. You can, however, give the same name to gates under different subpopulations. Later, you will see examples of how this is useful to more complex analyses.

FlowJo identifies subsets using the names of populations. When you later create graphical layouts or tables, you tell FlowJo that you want information about a population (like CD45+ Lymphocytes). FlowJo will look within all of your samples for a subset or statistic with this name (and ancestry) to extract the information you want. Importantly, the precise Lymphocyte or CD45+ gate can be different between different samples, but FlowJo will still recognize the populations and get the data you want from them. In fact, you can even draw a gate on completely different parameters – but if you name it Lymphocytes and it is at the sample level, then FlowJo assumes you have selected the same *kind of cells* as every other Lymphocytes gate attached at the sample level.

In conclusion: The name of a subpopulation is the fundamental name by which FlowJo identifies subsets of cells.

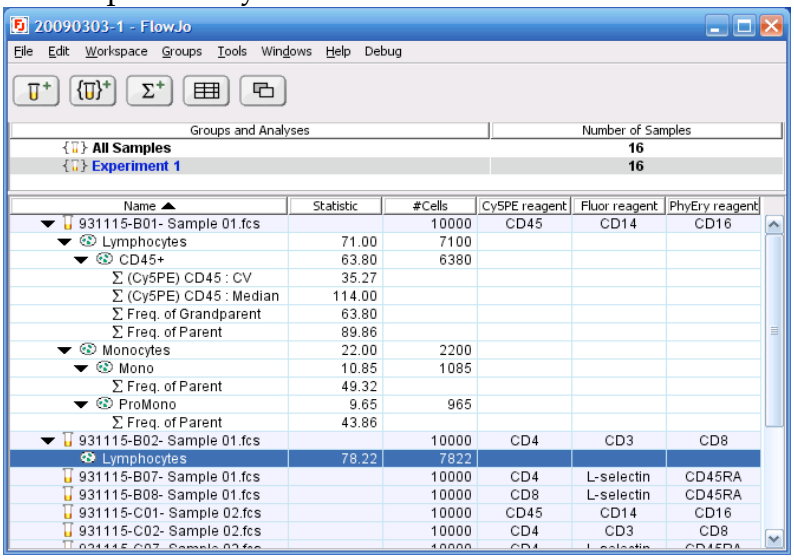
Lesson 3: Copying Analyses to Other Samples

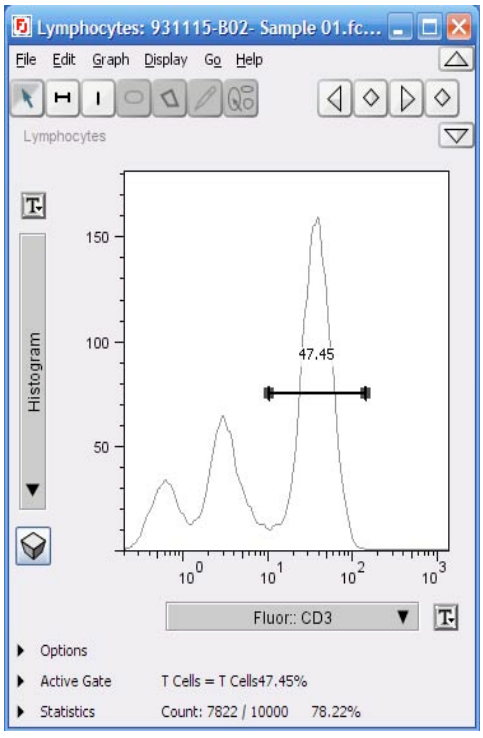
One of the basic principles of FlowJo is that virtually all analyses that you perform on a population of cells (a sample or a subset) can be applied to other populations using the drag-and-drop feature. Hence, when you click on an analysis (for instance, a statistic or a gate creating a subset) and you drag it to another place in the Workspace, FlowJo copies the analysis into the new location.

Now, it is time to analyze another set of cells from this same patient. This lesson builds on the Workspace you finished in Chapter 2; alternatively, you can open the workspace named Tutorial WS (Chapter 3).wsp Browse to the folder Experiment 1 and select the first sample. Click OK and the workspace will be populated with 16 samples; four sets of PBMC, with four stains each. We will analyze the second file in the Workspace, which has the sample from patient one stained with the second combination of reagents.

Here is where you will get the first glimpse of the powerful batch analyses that FlowJo can perform. You have already made a lymphocyte gate on stain 1; presumably, this same gate should be applied to stain 2. To do this, simply click on the Lymphocyte subset in the Workspace window, and drag it down so that the second sample (9931115-B02-Sample 01.fcs) is highlighted; then, let go of the mouse. FlowJo creates a new subpopulation, using the same gate you had previously created.

Your Workspace window now looks like the one on the right.


20090303-1 - FlowJo
File Edit Workspace Groups Tools Windows Help Debug
Groups and Analyses
Number of Samples
All Samples 16
Experiment 1 16
Name Statistic #Cells Cy5PE reagent Fluor reagent PhyEry reagent
931115-B01- Sample 01.fcs 10000 CD45 CD14 CD16
Lymphocytes 71.00 7100
CD45+ 63.80 6380
Σ (Cy5PE) CD45 : CV 35.27
Σ (Cy5PE) CD45 : Median 114.00
Σ Freq. of Grandparent 63.80
Σ Freq. of Parent 89.86
Monocytes 22.00 2200
Mono 10.85 1085
Σ Freq. of Parent 49.32
ProMono 9.65 965
Σ Freq. of Parent 43.86
931115-B02- Sample 01.fcs 10000 CD4 CD3 CD8
Lymphocytes 78.22 7822
931115-B07- Sample 01.fcs 10000 CD4 L-selectin CD45RA
931115-B08- Sample 01.fcs 10000 CD8 L-selectin CD45RA
931115-C01- Sample 02.fcs 10000 CD45 CD14 CD16
931115-C02- Sample 02.fcs 10000 CD4 CD3 CD8

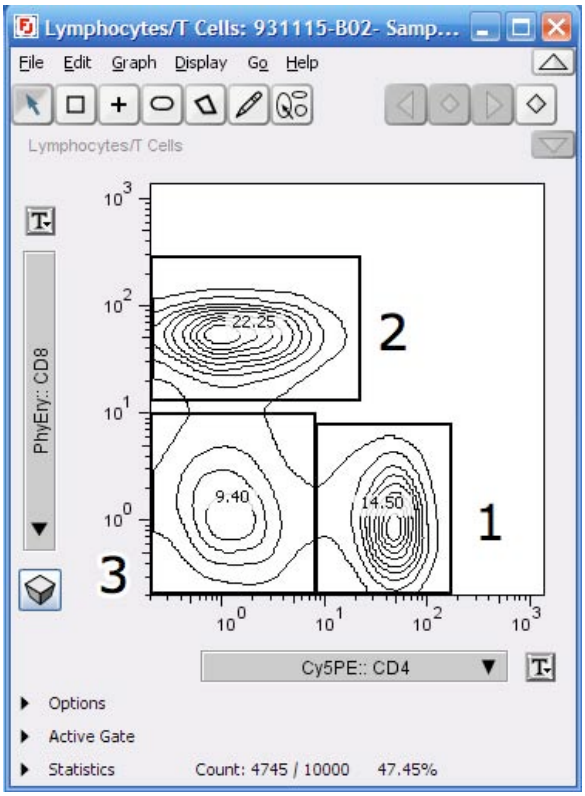


Double-click on this Lymphocytes subpopulation to open a graph of the lymphocytes within the 931115-B02-Sample 01 file.

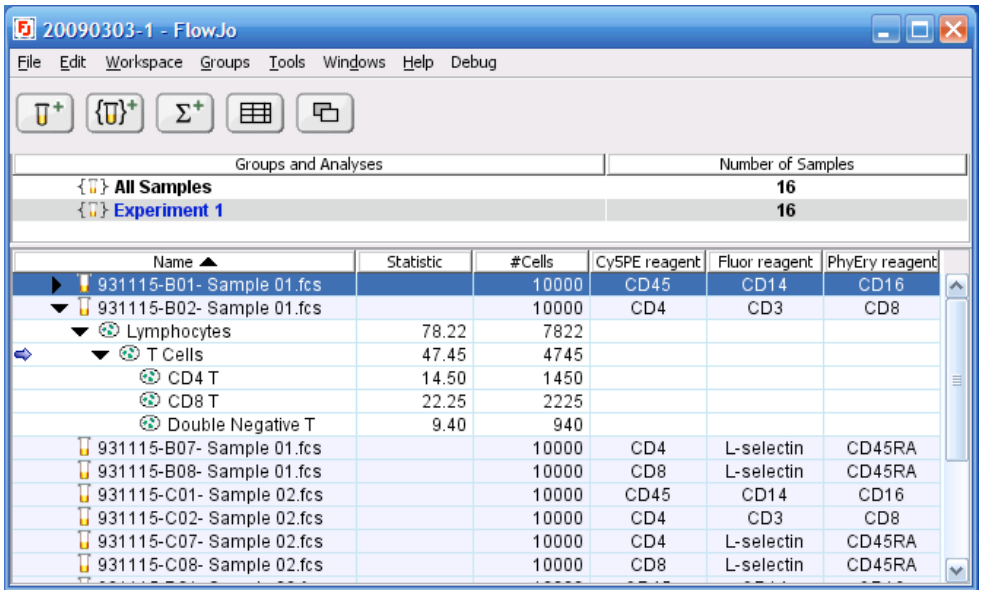
Now, change the axis labels to view a histogram of CD3. Create a gate on CD3+ cells, and name it T cells. At this point, your Graph window should look like the example on the left.

Click on the Down-arrow to open a graph of the T cell subset. Change the Y axis to PhyEry::CD8, change the X axis to Cy5PE::CD4. Create three rectangular gates:

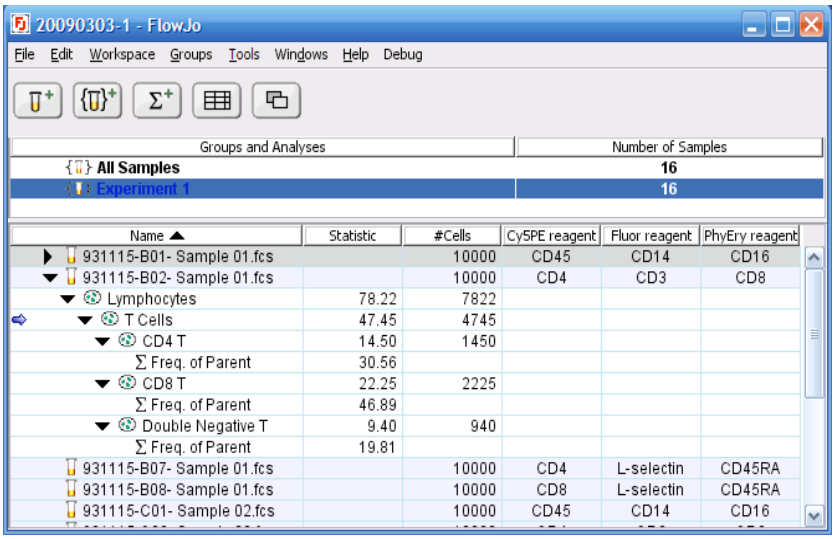
1. CD4 single-positives,
2. CD8 single-positives,
3. CD4- negative, CD8-negative (double-negative) T cells, as shown to the right.



In the ***Workspace*** window, click on the triangle next to the 931115-B02-Sample 01.fcs line. These triangles open and close the views of the subset hierarchy, much like you can do in the explorer window. In the genealogical terminology that FlowJo uses, the CD4 T, CD8 T, and Double Negative T subpopulations are siblings, and are children of the T cells subpopulation, grand-children of the Lymphocytes subpopulation, etc. Remember that the frequencies and cell counts shown to the right are with respect to the parent sample. The ***Workspace*** window (below) reflects this hierarchy.


20090303-1 - FlowJo
File Edit Workspace Groups Tools Windows Help Debug
Groups and Analyses
Number of Samples
All Samples 16
Experiment 1 16
Name Statistic #Cells Cy5PE reagent Fluor reagent PhyEry reagent
931115-B01- Sample 01.fcs 10000 CD45 CD14 CD16
931115-B02- Sample 01.fcs 10000 CD4 CD3 CD8
Lymphocytes 78.22 7822
T Cells 47.45 4745
CD4 T 14.50 1450
CD8 T 22.25 2225
Double Negative T 9.40 940
931115-B07- Sample 01.fcs 10000 CD4 L-selectin CD45RA
931115-B08- Sample 01.fcs 10000 CD8 L-selectin CD45RA
931115-C01- Sample 02.fcs 10000 CD45 CD14 CD16
931115-C02- Sample 02.fcs 10000 CD4 CD3 CD8
931115-C07- Sample 02.fcs 10000 CD4 L-selectin CD45RA
931115-C08- Sample 02.fcs 10000 CD8 L-selectin CD45RA

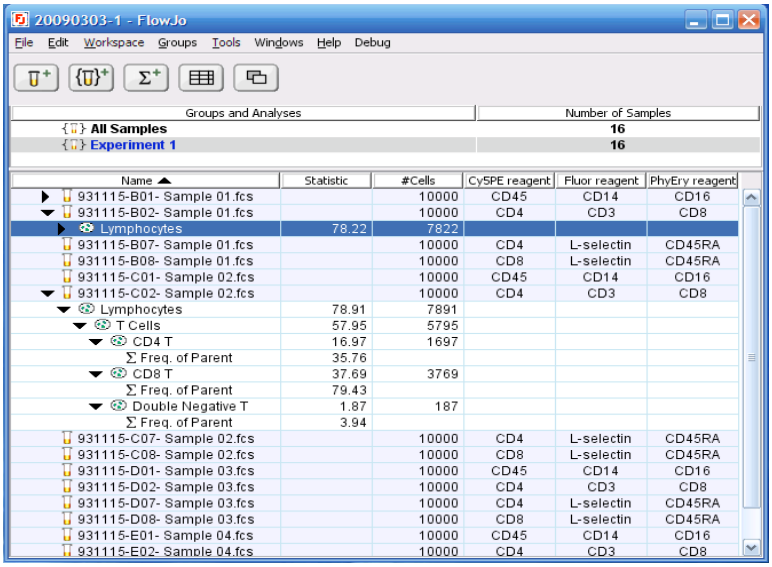
Add the Freq. of Parent statistic to each subset. You may also add it to the first subset (CD4 T) using the **Add Statistic** button, and then just click and drag the new row to the other subsets to save time (i.e., you are copying analyses from one population to another). Note that dragging a statistic from one population to another is a way of copying the mathematical operation. See the example on the next page. You could also select all three subsets (***shift***-click them), and add the statistic to all at once from the ***Statistics*** window.


20090303-1 - FlowJo
File Edit Workspace Groups Tools Windows Help Debug
Groups and Analyses
Number of Samples
All Samples 16
Experiment 1 16
Name Statistic #Cells CySPE reagent Fluor reagent PhyEry reagent
931115-B01- Sample 01.fcs 10000 CD45 CD14 CD16
931115-B02- Sample 01.fcs 10000 CD4 CD3 CD8
Lymphocytes 78.22 7822
T Cells 47.45 4745
CD4 T 14.50 1450
Σ Freq. of Parent 30.56
CD8 T 22.25 2225
Σ Freq. of Parent 46.89
Double Negative T 9.40 940
Σ Freq. of Parent 19.81
931115-B07- Sample 01.fcs 10000 CD4 L-selectin CD45RA
931115-B08- Sample 01.fcs 10000 CD8 L-selectin CD45RA
931115-C01- Sample 02.fcs 10000 CD45 CD14 CD16

A second patient sample stained with the same reagents is found a few rows down, labeled 931115-C02-Sample 02. Again, we would like to apply exactly the same gates and statistics performed on the first patient sample to the second. We could drag each line one by one to the second sample. However, FlowJo provides a special mechanism for copying entire analysis trees: if you hold down the ***ALT*** key as you drag with the **left** mouse button, FlowJo will take the subpopulation you clicked, as well as all of its descendants. You will see this occurring via the shaded box that FlowJo draws, which denotes all of the analyses that you are copying.

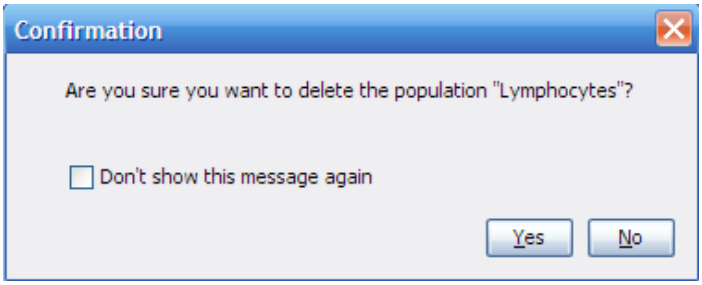
Click on the Lymphocyte gate, press the ***ALT*** key, and drag it to the C02 row. (Even though the children of the first lymphocyte gate are hidden if you close the triangle, they are still copied). Your workspace will now reflect the fact that you copied 8 different analyses (gates and statistics) with one operation.

There are more complex ways of selecting which analyses to copy. For instance, you can ***shift***-click several analyses to drag multiple different gates simultaneously or you can use the ***control*** key to select several subsets and/or statistics to be dragged.


20090303-1 - FlowJo
File Edit Workspace Groups Tools Windows Help Debug
Groups and Analyses
Number of Samples
All Samples 16
Experiment 1 16
Name Statistic #Cells CySPE reagent Fluor reagent PhyEry reagent
931115-B01- Sample 01.fcs 10000 CD45 CD14 CD16
931115-B02- Sample 01.fcs 10000 CD4 CD3 CD8
Lymphocytes 78.22 7822
931115-B07- Sample 01.fcs 10000 CD4 L-selectin CD45RA
931115-B08- Sample 01.fcs 10000 CD8 L-selectin CD45RA
931115-C01- Sample 02.fcs 10000 CD45 CD14 CD16
931115-C02- Sample 02.fcs 10000 CD4 CD3 CD8
Lymphocytes 78.91 7891
T Cells 57.95 5795
CD4 T 16.97 1697
Σ Freq. of Parent 35.76
CD8 T 37.69 3769
Σ Freq. of Parent 79.43
Double Negative T 1.87 187
Σ Freq. of Parent 3.94
931115-C07- Sample 02.fcs 10000 CD4 L-selectin CD45RA
931115-C08- Sample 02.fcs 10000 CD8 L-selectin CD45RA
931115-D01- Sample 03.fcs 10000 CD45 CD14 CD16
931115-D02- Sample 03.fcs 10000 CD4 CD3 CD8
931115-D07- Sample 03.fcs 10000 CD4 L-selectin CD45RA
931115-D08- Sample 03.fcs 10000 CD8 L-selectin CD45RA
931115-E01- Sample 04.fcs 10000 CD45 CD14 CD16
931115-E02- Sample 04.fcs 10000 CD4 CD3 CD8

The workspace now appears as shown on the right. You can delete analyses by selecting them and pressing the ***delete*** key. Select the lymphocyte subpopulation that you just created, and press the ***delete*** key. When you delete the lymphocyte gate, all of the subsets of lymphocytes will also be deleted– this is because those subsets have no meaning without a lymphocyte gate being

present. Remember, every subset that you name is, in reality, a subset defined by all of the gates of its ancestors - the parent gate, grandparent gate, etc. When you delete a gate, FlowJo lets you know that all of its descendants will also be deleted in the confirmation request.



One more detail: when you create a subset, the gate used to create that subpopulation remembers both the parameters *and* the stains on which the gate was drawn. In other words, the CD4 and CD8 T cell gates drawn above were created on a sample that had PE CD8 and Cy5PE CD4. For this (and other) reasons, it is important that you carefully enter the appropriate stain names when you collect the samples. Naming stains appropriately is good practice anyway – you will then always have properly annotated data.

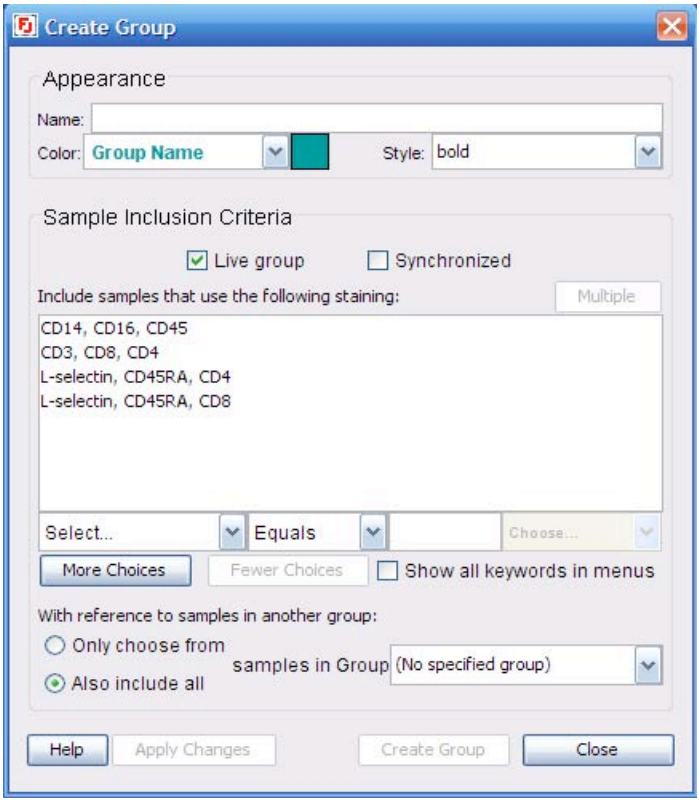
Of course, another good practice is to save your workspace files. The workspace does not have to be saved in the same location (or even same disk) as the data files; the workspace remembers where the data files are.

Lesson 4: Groups and Batch Analyses

In this lesson, you will learn how to take advantage of sample groups. Groups are the primary mechanism by which FlowJo allows you to perform batch analysis. You may create as many groups as you wish; each group can consist of any of the samples within the Workspace (and samples may belong to more than one group). In general, doing something to a group will be equivalent to doing the same thing to every sample in that group—the advantage is that you do it all at once. Remember that the default All Samples group always contains all samples in the workspace. Only add analyses to the All Samples group if you want them to be applied to all the samples in the Workspace.

In Lesson 3, you learned that by dragging multiple nodes, or entire trees of nodes, you can accomplish the first step in batch analysis: the ability to replicate multiple analyses simply, and with the guarantee that the copies are identical to the originals. But that still leaves the next step: how can these analyses be applied to a whole set of samples at once? To accomplish this, FlowJo allows you to create groups of samples. A group, which is simply a list of samples from the Workspace, can then serve as a sample surrogate. That is, you can apply analyses to a group much as you can apply them to single samples.

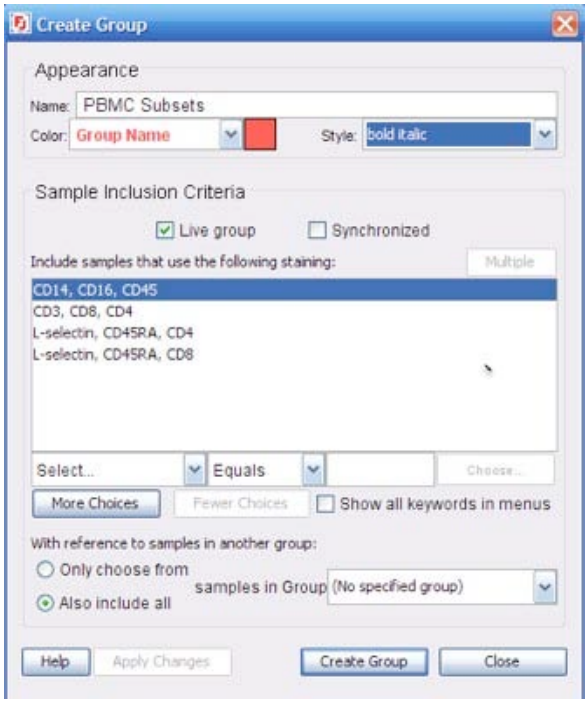
A group may consist of any number of samples from the Workspace. Any given sample can belong to as many groups as desired. However, there is one rule about groups and group analyses that is inviolate: every sample in a group has every gate, statistic, or other analysis specified by that group (as long as it is applicable to the sample). This will become clearer in the next few steps of the tutorial. This lesson builds on the Workspace you finished in Chapter 3. Or, open our premade workspace named ***Tutorial WS (Chapter 4).***



The first step is to create a group that contains a set of samples which will receive similar kinds of analyses. In our case, we will begin by analyzing the samples stained with the second combination of antibodies (CD3, CD4, and CD8). In the Workspace, click on the second button in the button bar, **Create New Sample Group**. FlowJo brings up the window shown on the right. Using this window, there are a variety of ways to make new groups. The middle portion of the window lists every different combination of

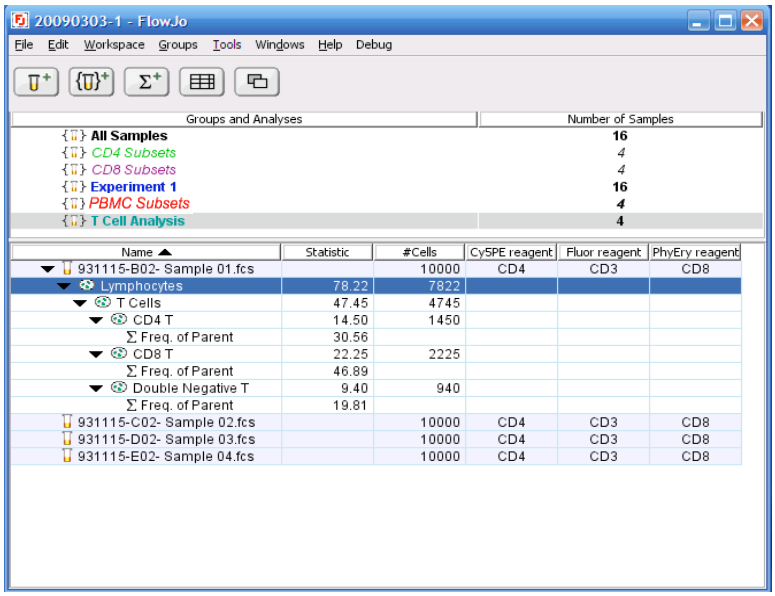
stains present in the whole list of samples. They are presented in channel order (in this case, FITC, PE, Cy5PE). Stains which were left blank at the time of collection would be noted with an ellipsis (…) Click on the CD3, CD8, CD4 combination, telling FlowJo to select all samples with this combination. Enter the name "T Cell Analysis" in the box at the top, and click on the **Create Group** button.

The window is still present (you will notice a new line in the Workspace window corresponding to the group you just created). You will now create your second group, corresponding to the analysis of the CD14, CD16, CD45 stains. Again, click on the stain combination, and enter the name for this group ("PBMC Subsets"). Before you create this group, change the color to red (click on the color box), and select Bold Italic from the Font Style popup menu. Your Create Group window should look as shown at the right.



Now click the Create Group button to create this group. Finally, make two more groups, called CD4 Subsets and CD8 Subsets, adding the third and fourth stain combinations, respectively, and set the color to green, and the style to italic. When you are finished creating groups, click Close. Click on the T Cell Analysis group; your Workspace window should look similar to the one below. When you select a group in the list of groups, its contents are displayed below in the samples list.

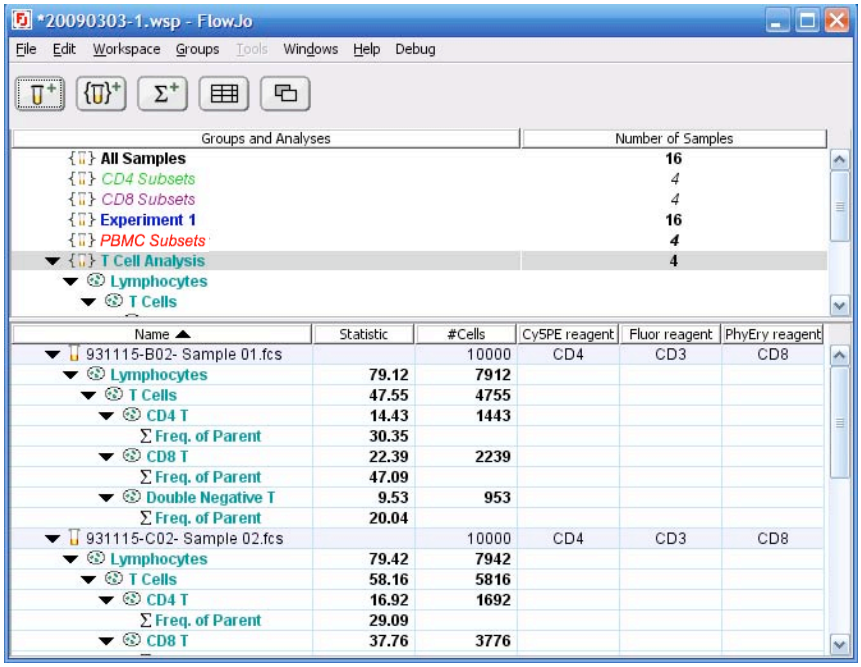
Each group is displayed in the upper (group) panel, in the color and style you have chosen.



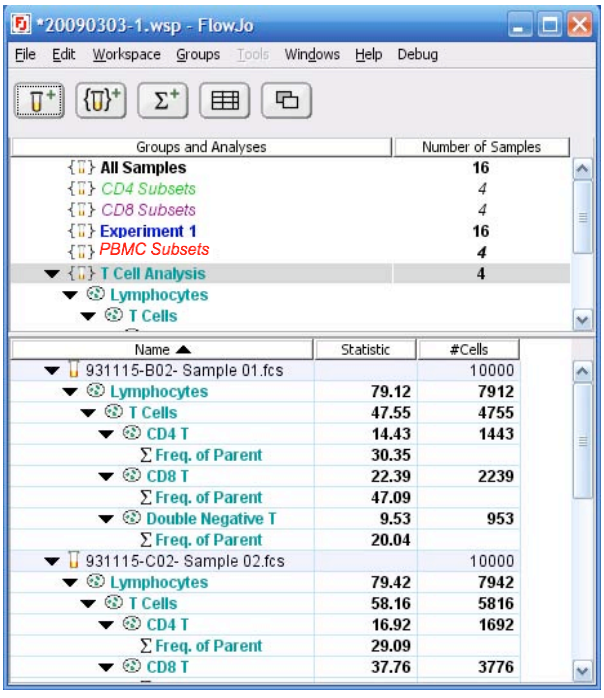
Right click and select **Group Properties** if you want to make changes. The number to the right of each group tells you how many samples are included in each group. Since there were four patient PBMC preparations, each stained with each combination of antibodies, each new group has four samples listed. Click on each of the groups, and verify that a different set of four samples is present in each of the different groups. Finish by selecting the T cell analysis group.

Remember that a group is a sample surrogate. Thus, any analysis that you apply to the group is applied to all of the samples in that group. To see this in action, click on the Lymphocytes gate in the lower **Samples** panel. Hold down the ALT key while dragging with the **left** button (so that the entire tree of analysis is copied), and release the mouse when it is above the **T cell analysis** group in the upper part of the window.

Notice that several things have happened. First, the analysis tree has been added to a row indented beneath the group name as if it were a sample. Second, the analyses have been added to every sample in the sample panel. Third, all of these analyses now appear in **teal and bold**, which is the same color and style that you defined for this group. You can see all of these in the example workspace on the right.

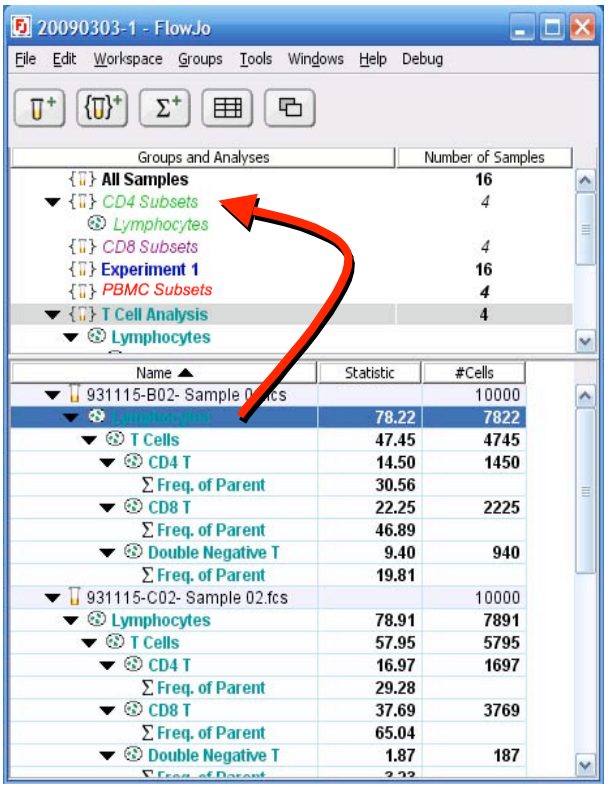


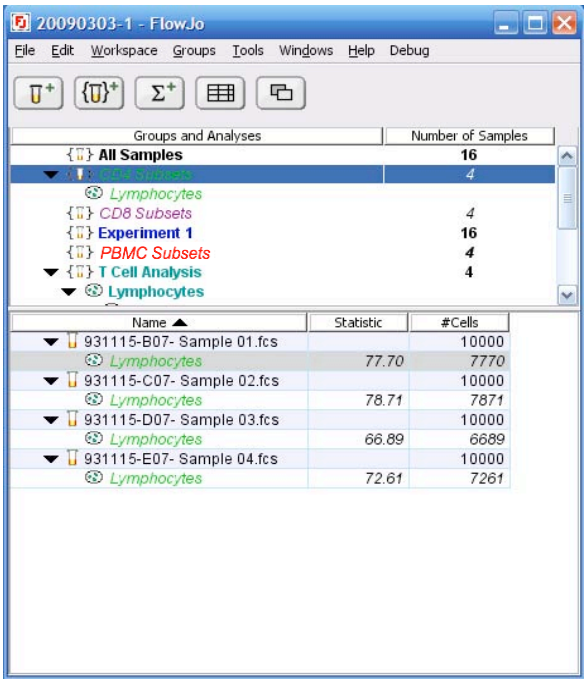
The color/style annotation of the analyses is an important cue for you to note. Any analysis that appears in the Workspace in the color and style of the group is guaranteed to be exactly the same as the group's version. Thus, the gate will be in exactly the same location, applied to the same parameters, etc. This is how you can guarantee that the exact same analyses have been done on all of your samples. (We will see later how to modify analyses for particular samples).



Use the menu item **Workspace > Edit Columns...** to remove the reagents from the workspace to simplify our view. You can choose to display or hide whatever attributes you prefer.

Now, we move on to the next set of analyses, the CD4 and CD8 subsets. Again, we will use groups to speed up the process (and ensure that identical gates are used for common gatings, like Lymphocytes). While the CD4 and CD8 subset analyses used different stains, we can still copy the lymphocyte gate, since it is based only on Forward and Side scatter. Click on the Lymphocyte gate from one of the samples (e.g., the first one) in the currently selected group, and drop it on top of the CD4 subset group. The gate is now applied to all of the CD4 subset samples.

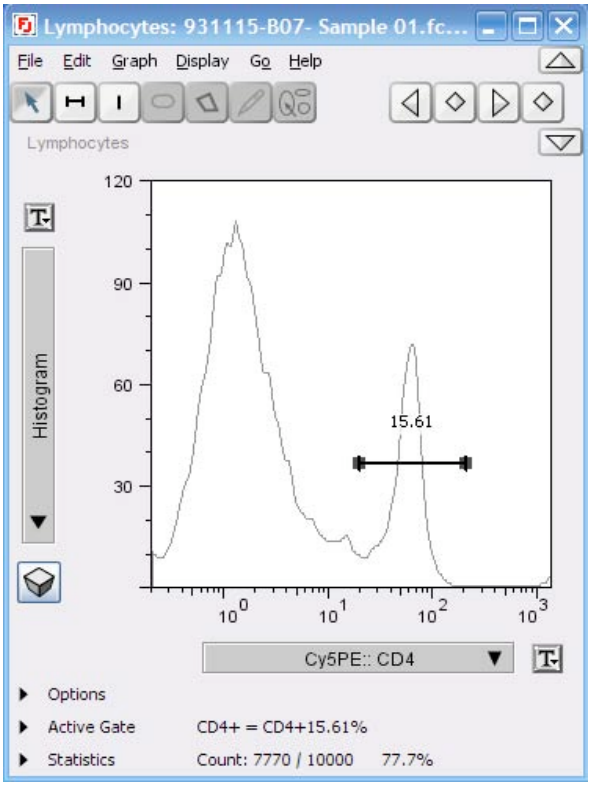


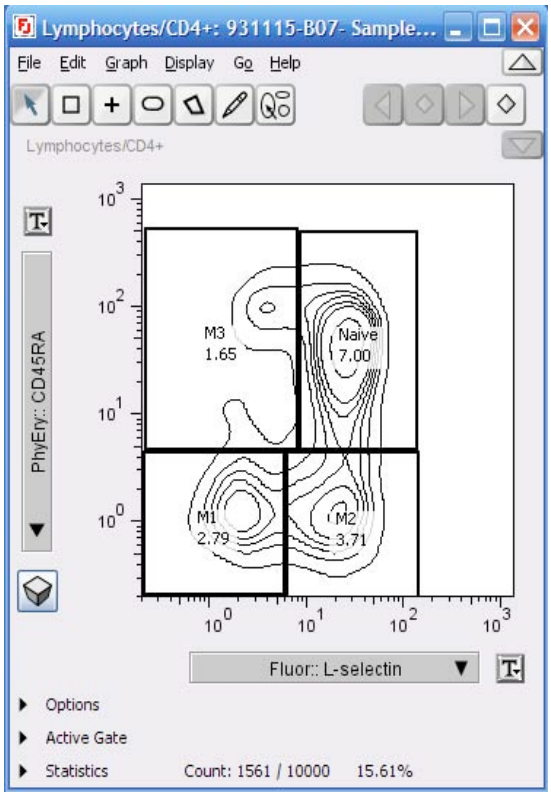


When you click on the CD4 subset **group**, you will see the four samples in the lower **Sample** panel with the lymphocyte gate (now drawn in green italic), as shown on the left.

Double-click on the first sample's lymphocyte subset to open its graph, and change the graph to show a histogram of CD4. Using the range gate tool, create a gate for the CD4+ T cells like the one shown right.

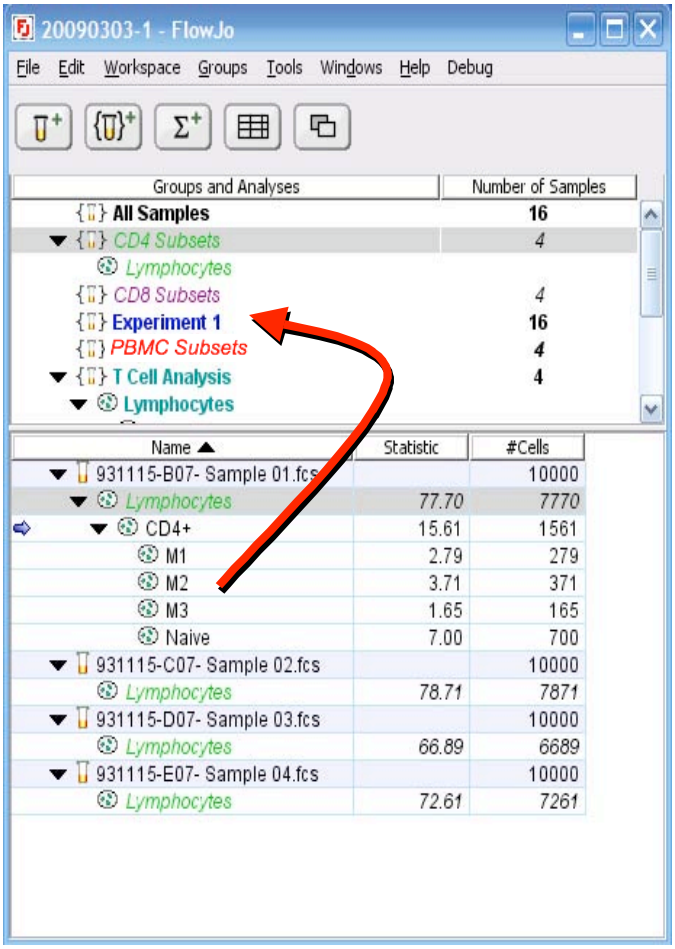
Click on the Down-arrow in the box at the upper right corner of the Graph Window, in order to view the population defined by the active gate. Now you are looking at the CD4+ subset within the lymphocyte gate.





To show the graph on the left (but without gates), click on **Options** and select **Contour** as the graph type, and change the axes to CD45RA vs. L-Selectin (CD62L). You will see four populations (at least). Create a rectangular gate around these populations as shown in the figure left. The CD45RA+LSelectin+ cells are *naive*; the rest are memory cells. You can name them M1, M2, and M3.

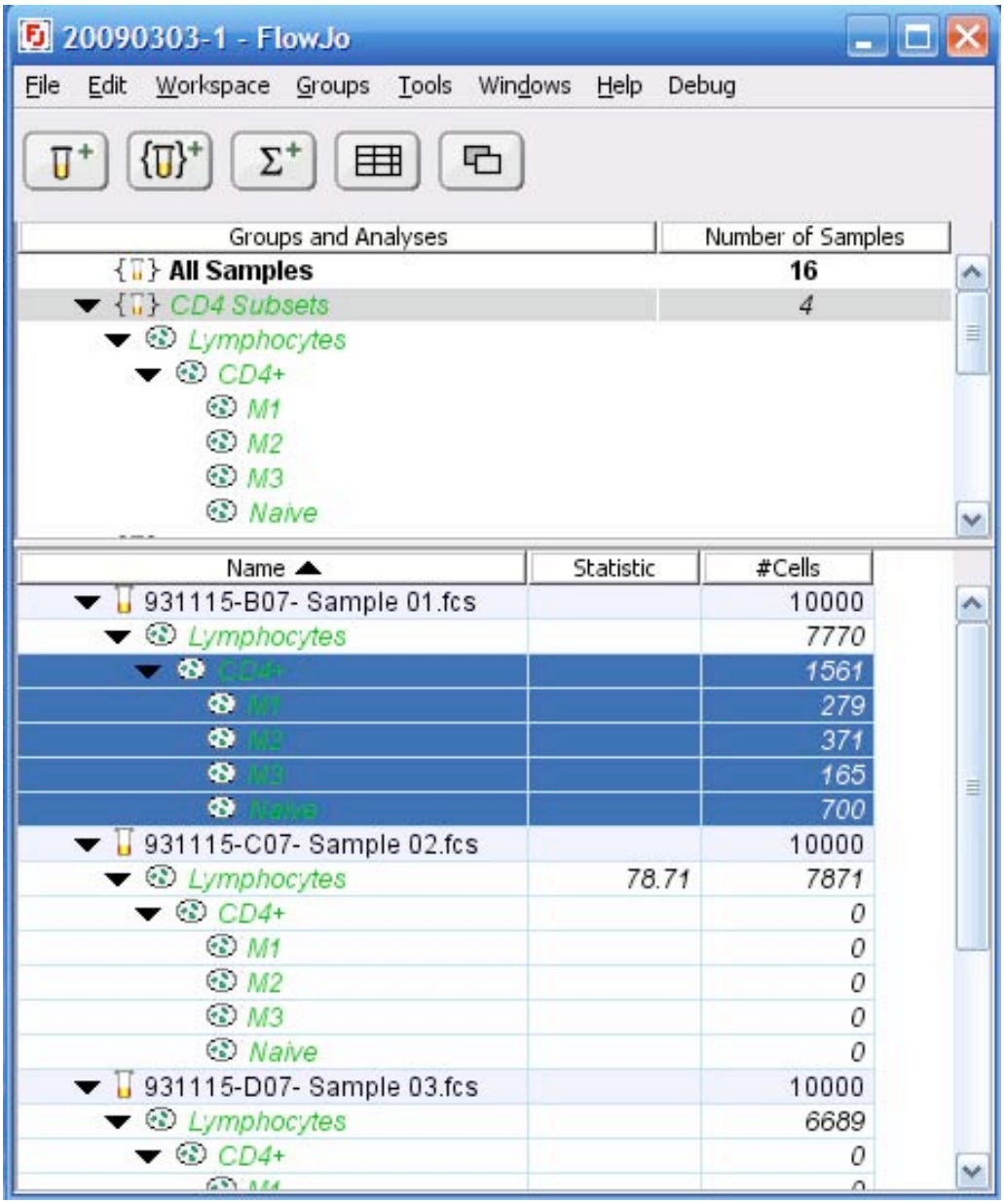
Your graph should now show these four gates. Close the ***Graph*** windows. Your Workspace will show the subsets you just created, including the differentiation subsets of CD4 cells, which are a subset of lymphocytes, which are a subset of the entire sample (see below).



Note that the CD4 gate (and the CD4 subset gates) are *not* in green. This is because these gates have not been applied to the group, and therefore are not identical to a group analysis. They are drawn in plain black to denote the fact that these analyses are unique versions belonging to the sample. The sample is in the group, but the gates have not yet been applied to the group.

To apply them to the group, click on the CD4 T subpopulation, and use the ***ALT***-drag action to move the gates onto the Lymphocyte subset of the **group**. (Note that if you were to drop it onto a higher level, a group name, the tree would be applied to each sample at the sample level – not to the lymphocyte gate. That is, the gates attach themselves to the row that they are dropped onto).

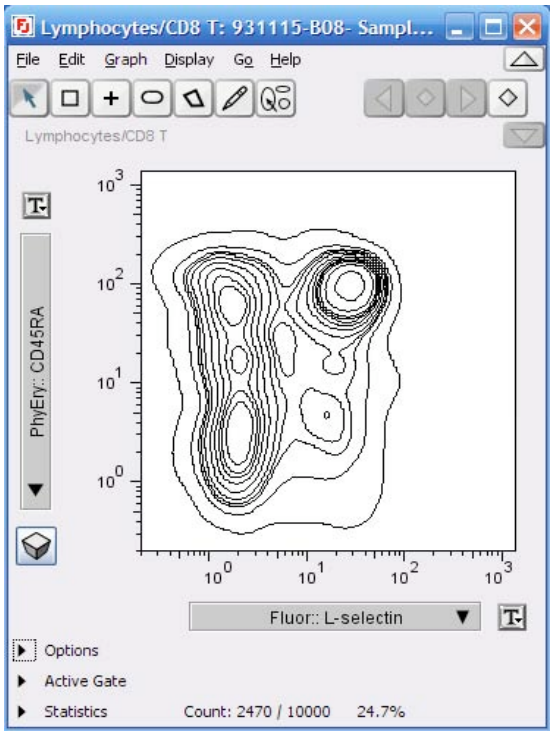
Your Workspace now shows these gates applied to all of the samples in the group, as shown here.



Lesson 5: Modifying Group Analyses

In this lesson, you will learn how to take advantage of previous work you have done (drawing gates, generating statistics) to save time when you do similar analyses. You will copy the gates you created for the CD4 cells to the CD8 cells. However, you will have to adjust them, then adjust the entire set of samples simultaneously, taking advantage of group analyses. Finally, you will finish all the group analyses for the entire sample experiment. You will then see how easy it is to modify the lymphocyte gate used by every sample with a single drag-and-drop action. Use your existing Workspace, or to better stay in sync with this text, open the workspace named ***"Tutorial WS (Chapter 5)".***

Let's move on to the CD8 analyses, taking advantage of what we have already accomplished.



1. Again, drag a lymphocyte gate (the single population only—don't select the child gates) from any of the samples in the **T Cell Analysis** samples panel and drop it onto the **CD8 Subset** analysis **group**.

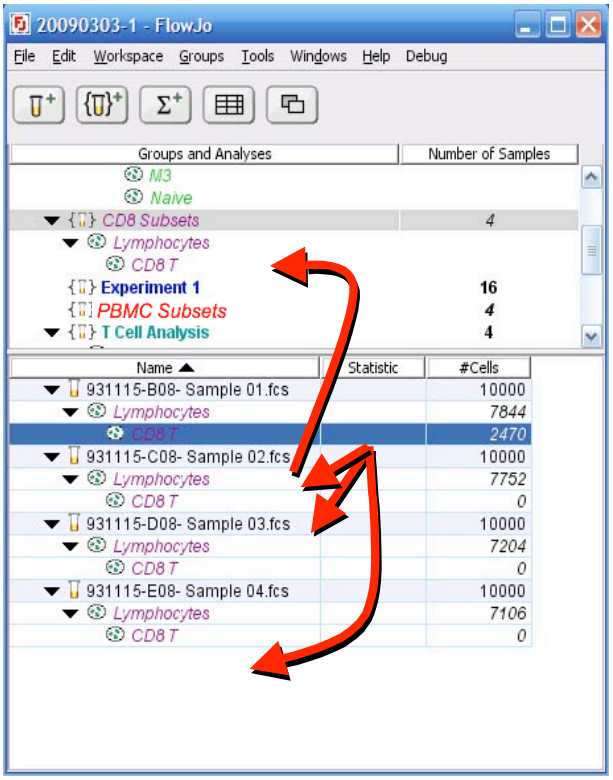
2. Select the group, and open the graph of the lymphocyte subpopulation from the first sample.

3. Create a **CD8 T** gate on the CD8 histogram (much as you did for CD4 cells).

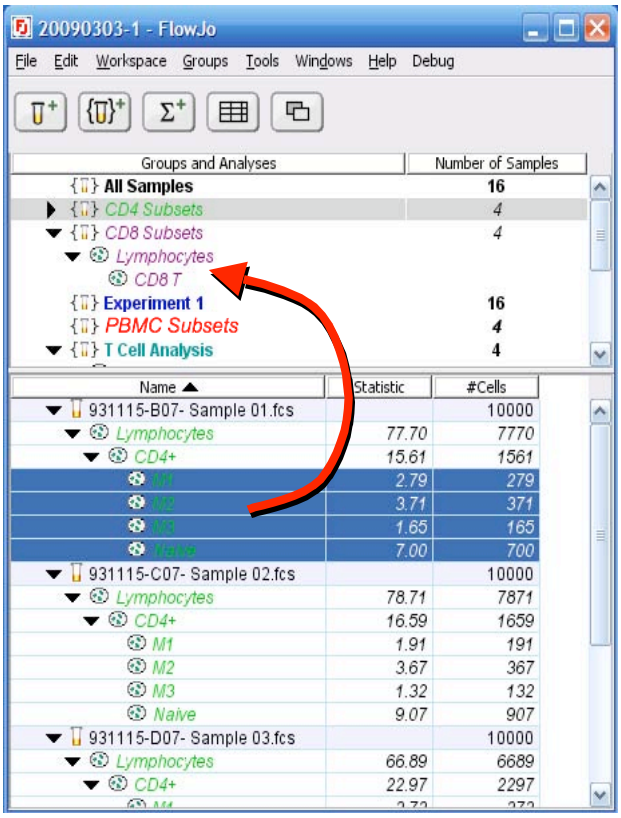
4. Then show the contour plot of CD45RA vs. L-Selectin, shown at the left.

5. Move this window to the side where you can still see it, and click on the Workspace window to bring it to the front.

6. Drag the CD8 T gate you created onto the group's lymphocyte gate; the ***Workspace*** window will show the group's CD8 gate under each of the member samples, as shown here to the right.

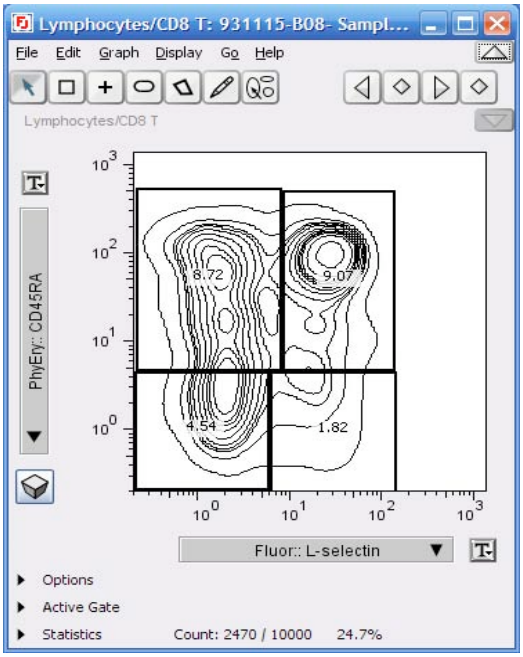


You will notice that the subsets of CD8 cells are similar to the subsets of CD4 cells. We will copy the same gates you used for the CD4 cells onto the CD8 populations; you will then fine-tune them.

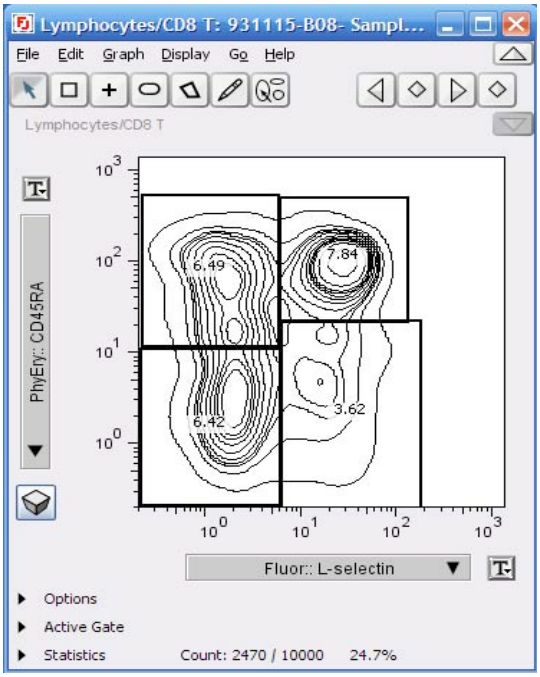


Watch the open ***Graph*** window as you do this: the four gates will appear in this window as they are attached to the sample.

Again, FlowJo makes sure that every window that is open will reflect any changes you make. The Graph window appears as shown below.



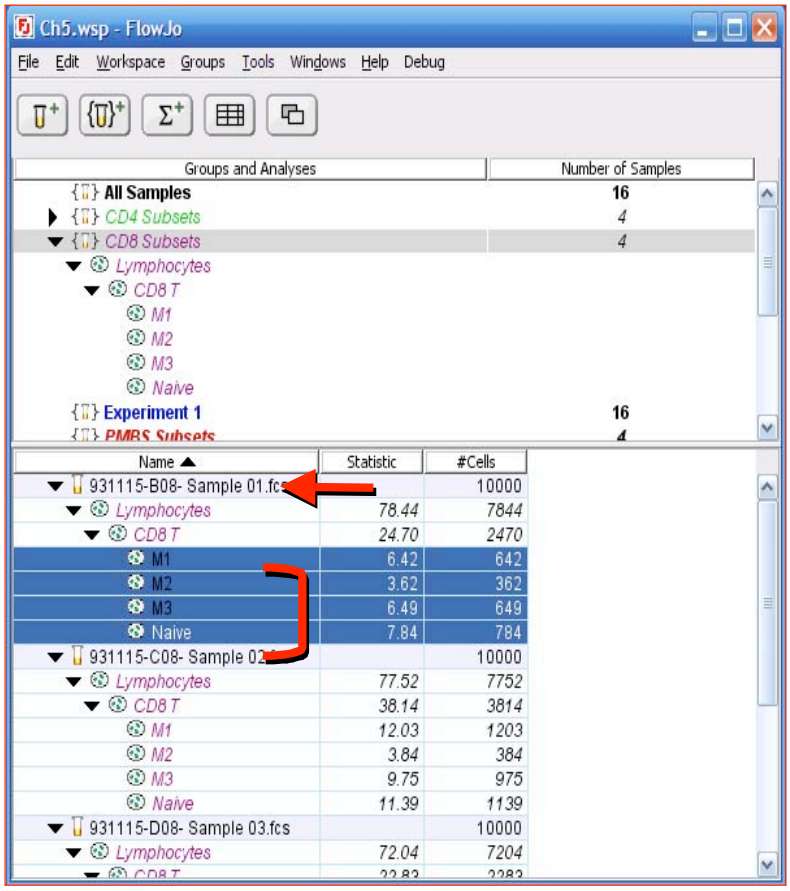
Select the CD4 group again. Now, ***shift***-click on the Naive, M1, M2, and M3 subpopulations from one of the samples (see left). Drag this set of gates, and drop it onto the CD8 T gate, in the CD8 subset analysis group.



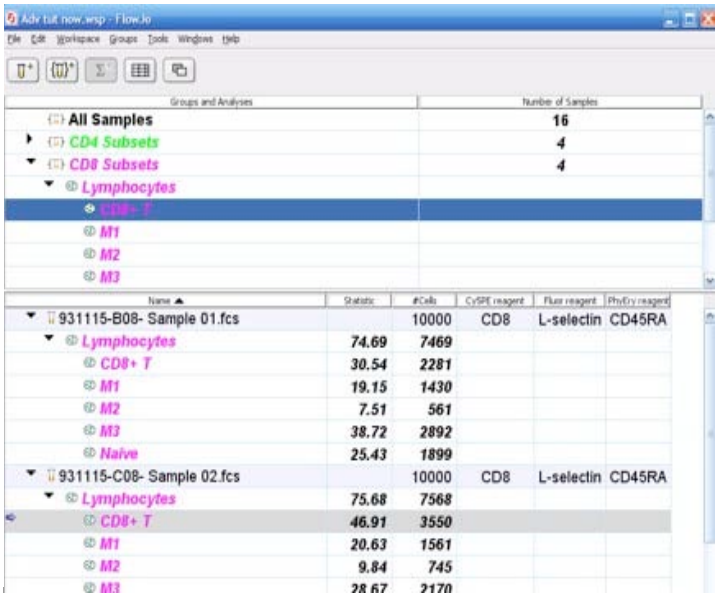
It is apparent that the gates are not positioned properly for CD8 T cells (these are the gates that were appropriate for CD4 subsets). In particular, they look as though they should all be moved up, since CD8 cells tend to express higher levels of CD45RA than CD4 cells. Click on each gate, and move them so that they more accurately enclose the populations, as shown left.

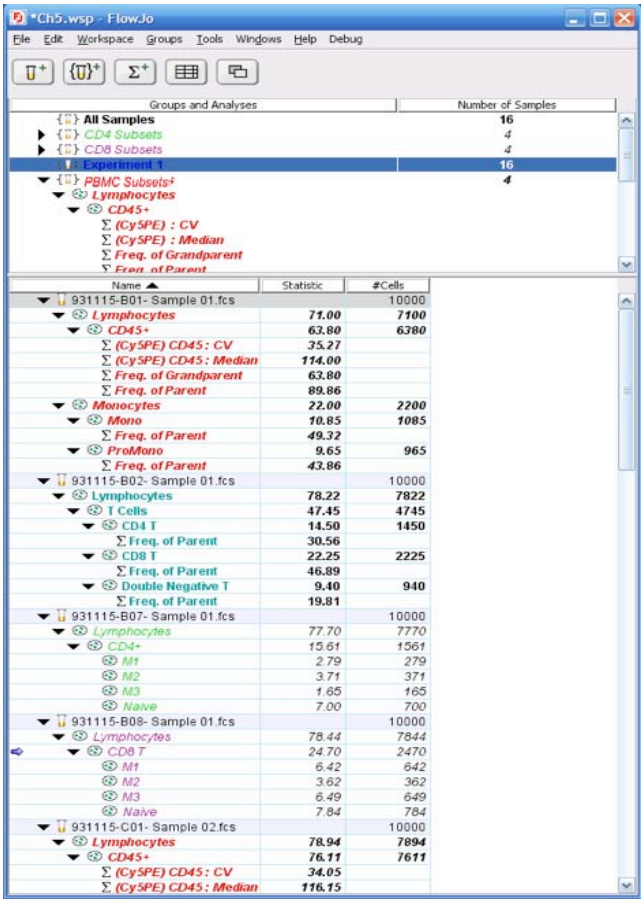
Leave this window open for now. Go back to the ***Workspace***, and open the graph for the CD8 subpopulation for Sample 02 (C08). It still has the gates copied from the CD4 stained sample.

Move this window off to the side as well, and watch it during the next operation. In the ***Workspace*** window, you will note that the subpopulations for the first sample are drawn in black – this is because you modified them and they are no longer equivalent to the versions in the group.



However, these are the ones you want to apply to the group. Once again, this is easy to do: shift-click the four subpopulations (Naive, M1, M2, and M3, under the first sample), and drop them on the CD8 T gate in the group. In the Graph window for the second sample you will see the gates move to the new positions; in the Workspace window, all gates are now drawn in pink, since all are now identical (see right).

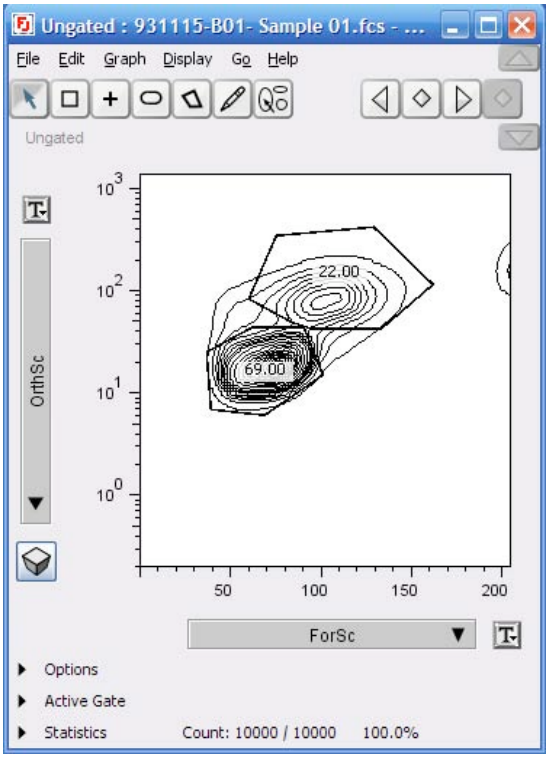




You are now finished with the T cell analyses; we will now go on to the PBMC subset group. ***Alt***-click and then drag first the Lymphocyte then the Monocyte gates from the first sample in Experiment 1 to the PBMC Subsets group. This applies the analyses to the entire group (see left).

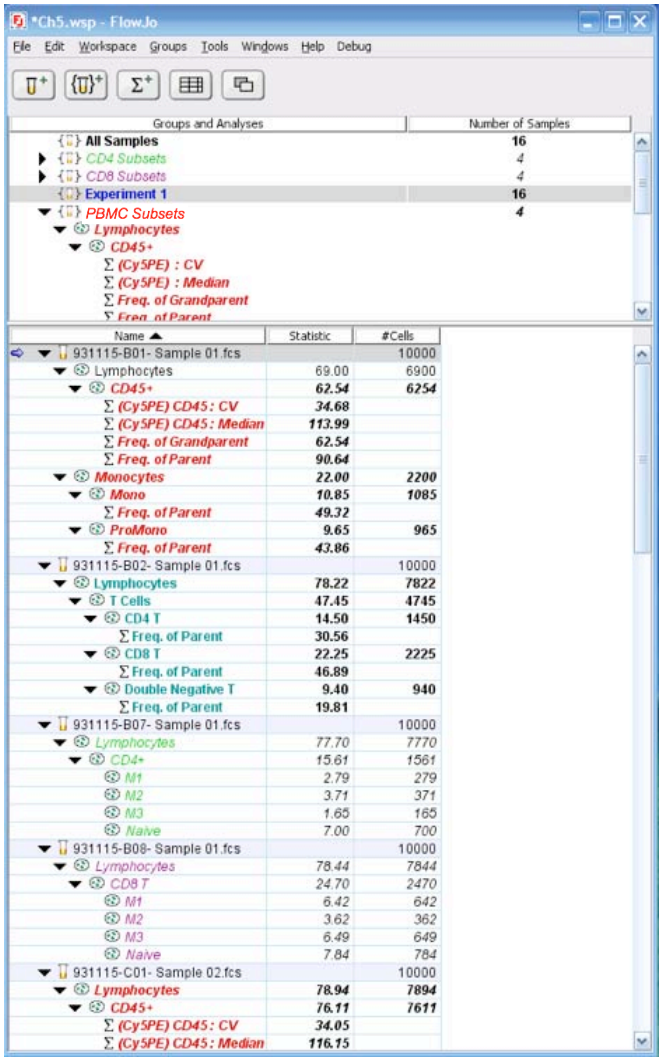
Now, if you click on the Experiment 1 group, you can look at all of the samples from this experiment in their full glory (left). Note that the assignment of color and style to the groups helps you identify which group any particular gate comes from.

As a final tune-up for the gating analysis, you will modify the lymphocyte gate and update all of the samples for this experiment to have the new version of the gate. It is important to see how the program's dynamic recalculation can be used to modify either single gates, or gates shared among multiple samples.

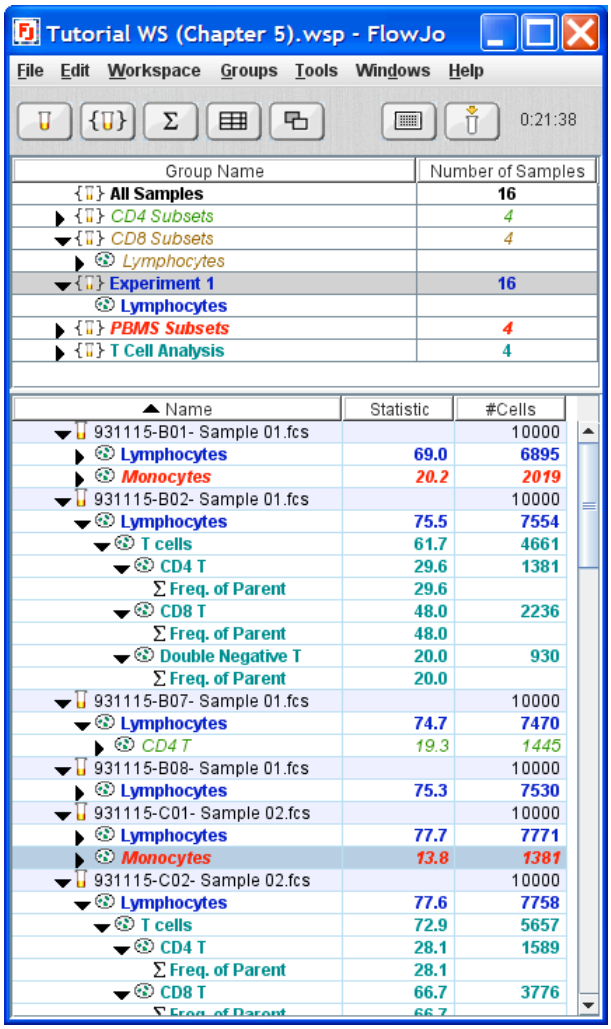


Open the graph of the very first sample, at the top level (in the sample list – double-click on the top line). You will see the two gates, the lymphocyte gate and monocyte gate. Select the lymphocyte gate, and drag the individual handles so that it is "tighter" around the population (see the graph on the left). Move all of the handles in to make this gate smaller.

Click the ***Workspace*** window; it will now reflect this modification by drawing the newly modified lymphocyte gate in black. (See the second line in the figure below).



How can we update all of the samples for this experiment? All of the samples for this experiment belong to the group Experiment 1, the group that was created when you dropped in the folder of FCS files. So, you can use this group for analyses that apply to all samples. (Alternatively, you could create a new group, select all samples, and drag them to the new group to add them, and then use that group). Drag the modified lymphocyte gate to the Experiment 1 group. Now look at the Workspace (shown on the next page).



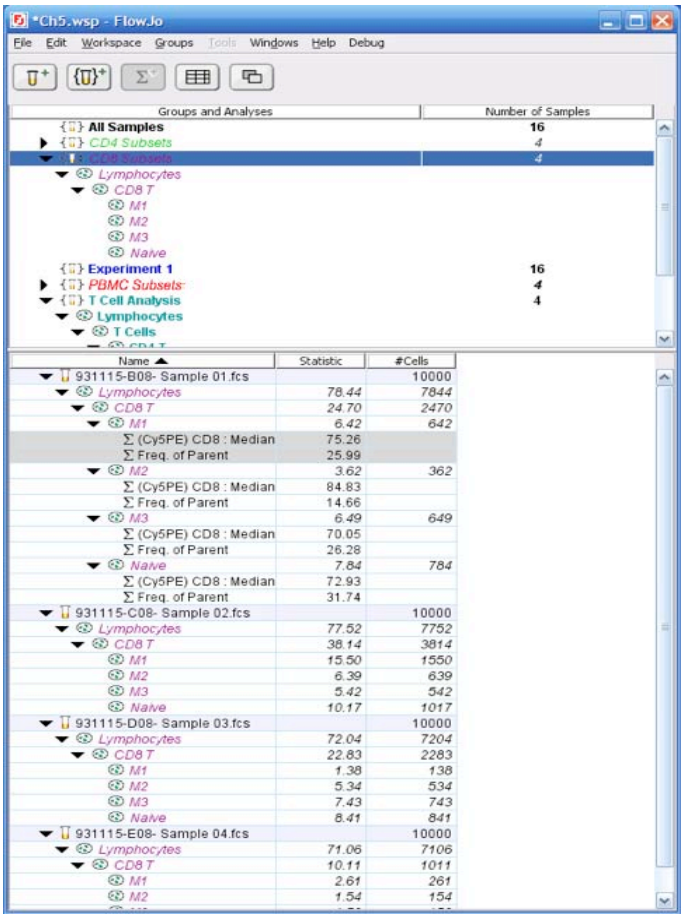
Now all Lymphocyte subsets are drawn in bold blue text, denoting that they are identical to the group's version. Open any sample's graph to verify that it has the right lymphocyte gate.

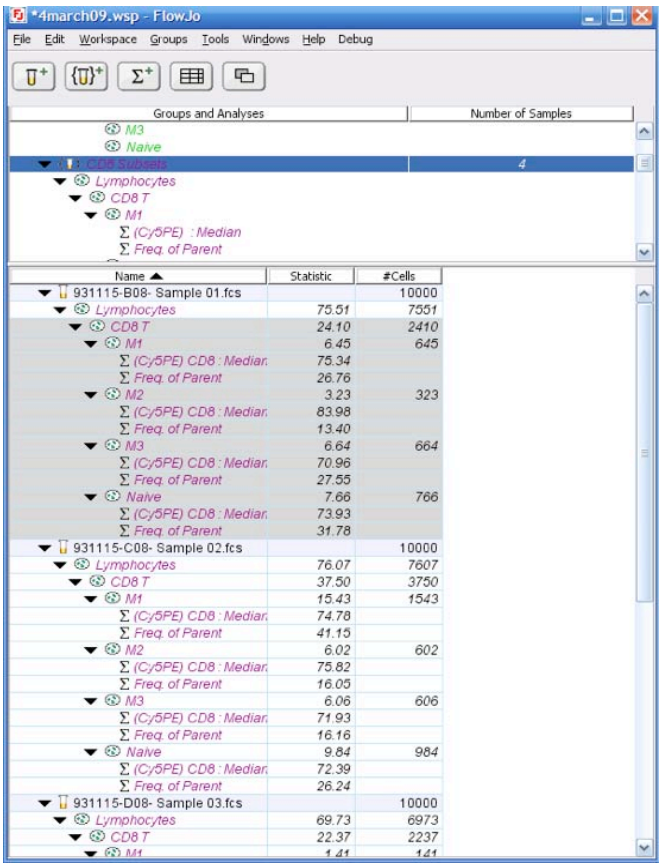
One final lesson before moving on to tabular and graphical presentations: merging analysis trees. Let's assume that you modify an existing analysis tree on a sample, for instance, to add several statistics to different subsets. You would now like to propagate these changes to the whole group (or other samples) without having to drag each new statistic individually. FlowJo gives you the ability to easily merge analysis trees that are very similar, adding only the new or updated analyses to the destination.

When you drag an entire analysis tree onto a population that already has parts of that tree, FlowJo assumes that you want to replace all of the existing analyses that have the same name.

As an example, select the CD8 Subsets group and add two statistics to the first M1 subset. Click on the **M1** subset, and click on the Add Statistic button. Ask for the **Median of Cy5PE (CD8)** and the **Frequency of Parent** statistic. Then, ***shift***-click these two rows in the Workspace, and drag them to each of the **M2, M3**, and **Naive** populations.

Here is an example where you have modified the existing tree (in sample B08), and wish to add the changes (the statistics) to C08. You could simply drag the two statistics nodes all the way to the **M1** subset with C08 (resulting in the workspace shown on the right). Then, you could continue this process. However, this is tedious, especially if you have more than a few changes. Instead, copy the whole tree.

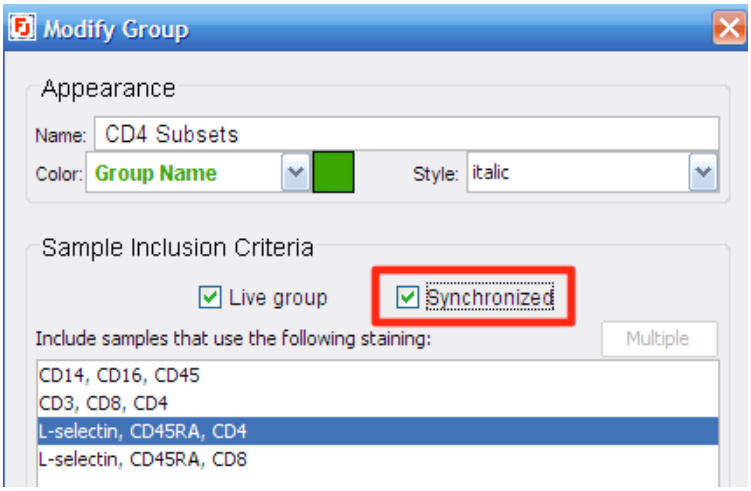




Click on the **Lymphocyte** subset under the sample named **93115-B08**, and, while holding the Alt key (to drag the entire tree), drop it onto the **C08** sample. Make sure you drop it onto the sample level – that is where it needs to be attached. You can also copy the tree to the CD8 Group to add the new statistics to all samples in the group. FlowJo assumes that when you drag an analysis tree to a group, you want to replace all of the existing analyses that have the same name. Then, every sample that has the group's version of those analyses will also get the new version that you have copied to the group.

Confirmation
The node 931115-C08- Sample 02.fcs already has a child with the same name.
Do you want to replace it?
Don't Ask Again
Yes
No

If you choose to **Synchronize Group's Gates**, FlowJo will automatically update the gates for all the samples in a group as soon as you adjust a gate on one sample. This option saves time by applying newly adjusted gates automatically to all the samples in the group; skipping the step of dragging the newly adjusted gate up onto the group name. However, it does not allow between-sample-variation of gates. All the gates will be the same as the last adjusted gate on any sample in the group.



Lesson 6: Tables and Layouts – Collating Data Output

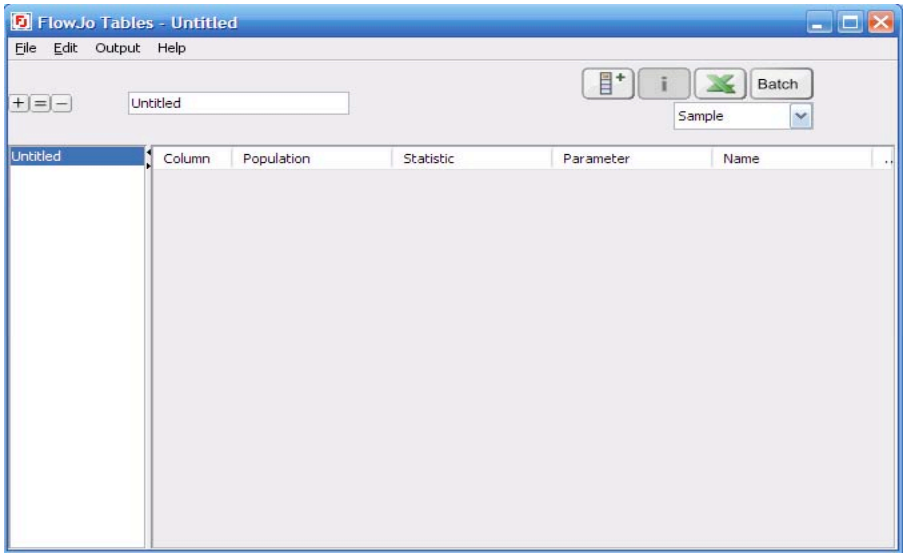
In this lesson, you will learn how to transfer any statistical analyses you have requested in the Workspace into other programs. You will be able to generate a table of statistics, bringing together any set of values from any combination of samples (using Groups to define the sample list).

FlowJo is not a comprehensive data presentation package. It provides the tools to analyze and graphically display flow cytometric data. Analysis tools such as linear regression, very complex graphical displays, etc., are more suited to programs specifically designed for those purposes.

However, FlowJo does provide tools to collate the output from multiple analyses so that you can import them into spreadsheets (tabular data) or into drawing programs (graphical displays). These tools include the ***Table Editor*** and the ***Layout Editor***.

Use your existing Workspace from Lesson 5, or open the tutorial workspace named "Tutorial WS (Chapter 6)."

To open the Table Editor, you can click on the fourth button in the button bar or select the Table Editor from the Windows menu. FlowJo shows you the Table Editor window as shown here. (Click in the title field and type a new name for this table).



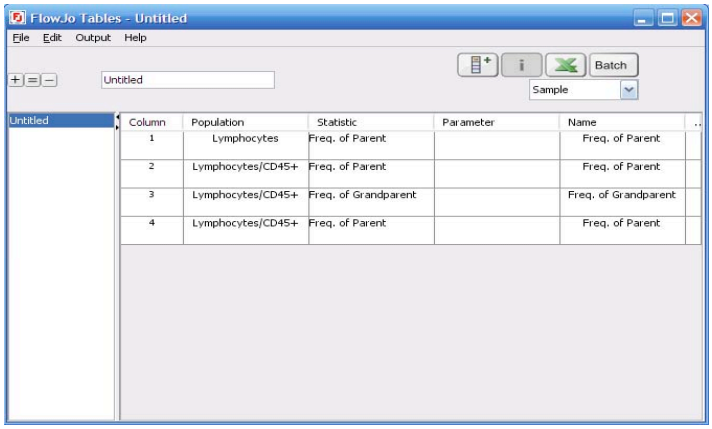
In this window, the left side holds the list of existing table templates. The right side will show the items in the currently- selected template.

What is a table **template**? When you define a table, you will add rows to a table template. Each row in the template defines one statistic that you want to export, such as frequency, mean fluorescence of FITC, etc. When you create the table, FlowJo cycles through each sample in the current group and requests the particular statistic defined in the table.

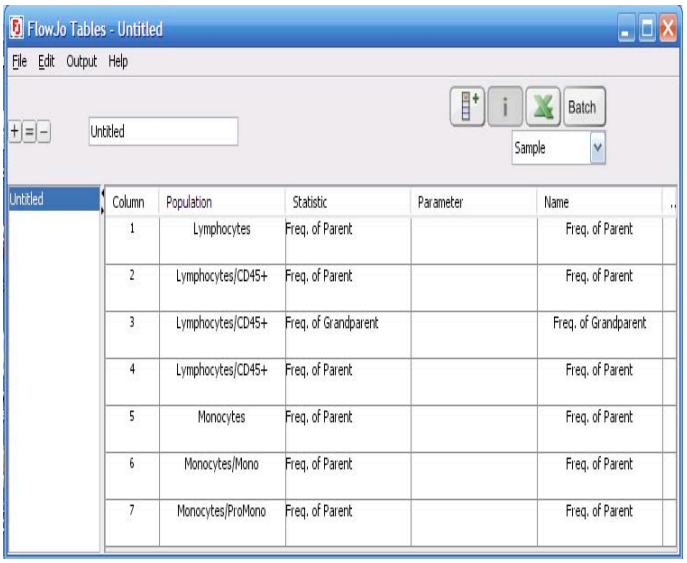
NOTE: ***When you create the table and import it into another program, you will have one row in the table for every sample in the current group, and one column for every statistic in the table template. Rows in the template create columns in the table.***

You may define as many table templates as you wish; you could use one for each different set of statistics. This one, as you may have guessed, will be used for generating a table of the major **PBMC** subsets analyzed in the first stain combination. Note that you can apply a template to any group; it doesn't matter which group was active at the time that you defined the table template. However, most likely your table will only be applicable to one group of samples.

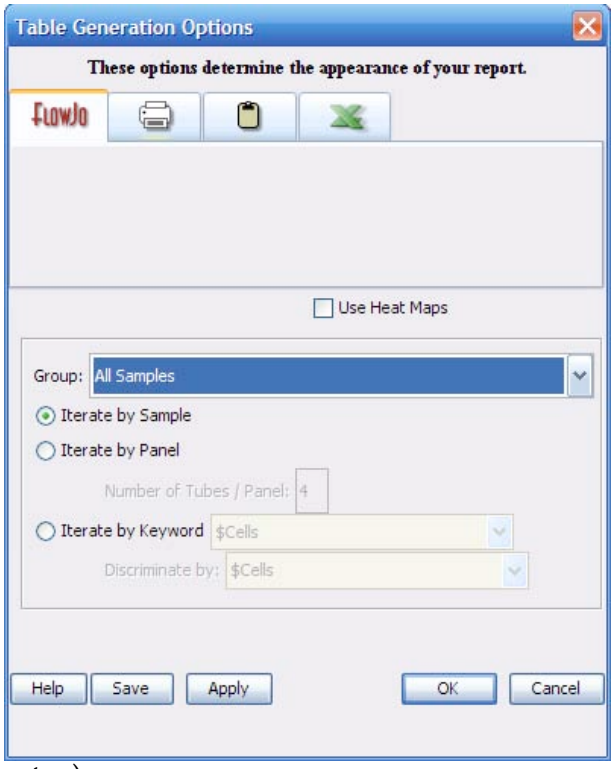
Go back to your Workspace, and select the **PBMC subsets** group. From the first sample only, select the **Lymphocytes** and **CD45+** subpopulations, and then the two frequency statistics under the "CD45+" subset. Drag the highlighted rows and drop them into the right portion of the ***Table Editor*** window. The ***Table Editor*** will now look like it does here.


FlowJo Tables - Untitled
File Edit Output Help
Untitled
Batch
Sample
Untitled
Column Population Statistic Parameter Name
1 Lymphocytes Freq. of Parent Freq. of Parent
2 Lymphocytes/CD45+ Freq. of Parent Freq. of Parent
3 Lymphocytes/CD45+ Freq. of Grandparent Freq. of Grandparent
4 Lymphocytes/CD45+ Freq. of Parent Freq. of Parent

Just as you can use the ***control select*** <u>then</u> drag action on analysis trees between samples, you can also use ***alt select <u>then</u> drag*** for transferring an entire tree to the ***Table Editor.***


FlowJo Tables - Untitled
File Edit Output Help
Untitled
Batch
Sample
Untitled
Column Population Statistic Parameter Name
1 Lymphocytes Freq. of Parent Freq. of Parent
2 Lymphocytes/CD45+ Freq. of Parent Freq. of Parent
3 Lymphocytes/CD45+ Freq. of Grandparent Freq. of Grandparent
4 Lymphocytes/CD45+ Freq. of Parent Freq. of Parent
5 Monocytes Freq. of Parent Freq. of Parent
6 Monocytes/Mono Freq. of Parent Freq. of Parent
7 Monocytes/ProMono Freq. of Parent Freq. of Parent

Click on the **Monocytes** gate in the first sample, and, while holding down the shift key, select any other subsets or statistics you wish to include. Then drag the selected items to the **Column Definition** window in the Table Editor. You will note that all of the analyses are added in order to the table (the ***Table Editor*** should now appear as shown left. When you drag a subset, a gate, to the table, FlowJo assumes that the statistic you want is the Frequency of parent of that subset.



To create the output table, turn iteration on by clicking on the Batch button near the top-right of the window. FlowJo will display the window shown left where you can select the group from which to build your table, as well as a keyword as alternative iteration source, etc. Select "Groups: PBMC subset" and Iterate by Sample. Click OK and FlowJo will display a new window in which the table will be shown. The finished table looks like that shown below.

At this point, FlowJo has cycled through every sample in the current group (there are four), and has calculated each of the nine different statistics you requested (on the applicable subsets). If a sample did not have the subset required, then there would be a blank entry in the table. (And, if the subset did not have the requested statistic node present, there would also be a blank entry).

Table - PMBC Subset Freq

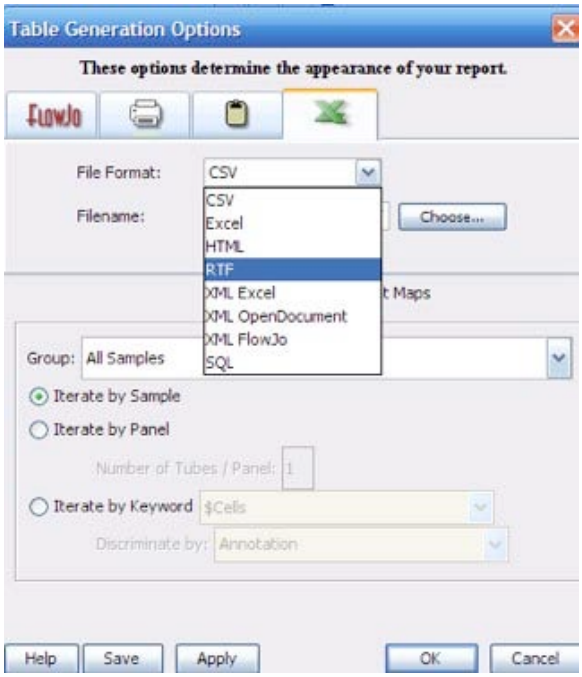
File Edit FlowJo Help

	Lymphocytes	Lymphocytes/CD...	Lymphocytes/C...	Lymphocytes/CD...	Monocytes	Monocytes/Mono	Monocytes/ProM...
Samples	**Freq. of Parent**	**Freq. of Parent**	**Freq. of**	**Freq. of Parent**	**Freq. of Parent**	**Freq. of Parent**	**Freq. of Parent**
931115-B01 - Sample 01.fcs	69.00%	91.17%	62.91%	91.17%	21.00%	47.00%	43.67%
931115-C01 - Sample 02.fcs	77.57%	96.96%	75.21%	96.96%	14.58%	39.51%	47.46%
931115-D01 - Sample 03.fcs	53.54%	92.87%	49.72%	92.87%	31.30%	54.06%	40.13%
931115-E01 - Sample 04.fcs	54.68%	91.77%	50.18%	91.77%	33.37%	49.36%	45.07%
Mean	**63.70%**	**93.19%**	**59.51%**	**93.19%**	**25.06%**	**47.48%**	**44.08%**
Standard Deviation	**11.62%**	**2.61%**	**12.12%**	**2.61%**	**8.84%**	**6.07%**	**3.06%**

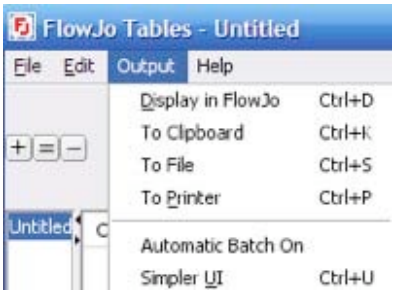
The column heads show you the name of the subset's parent gates ("ancestry"), the name of the subset, the statistic, and the parameter on which the statistic is calculated. Each row in the table corresponds to a sample in the current workspace. You may resize the columns by clicking on a column divider (in the table header) and dragging left or right.

The **Mean** and **Standard Deviation** of the statistics are displayed at the bottom of each column. When the summary statistics are computed, the numbers in the cells are drawn in bold italic if they are greater than one standard deviation away from the mean, and in red if they are over twice the standard deviation away from the mean. This will quickly highlight outlying samples making it easy to use the table editor to identify samples that are significantly different from the others in the group. You can change this formatting by clicking on the first icon in the table editor (shown right).

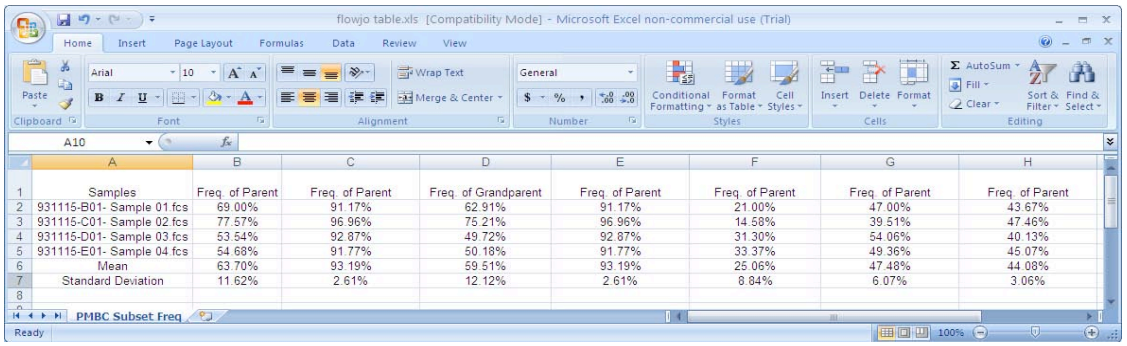
You can save the Table in different formats: CSV (Comma Separated Values), Excel, HTML (HyperText Markup Language), RFT (Rich Text Format), SQL (Structured Query Language) and different XML flavors (eXtended Markup Language). Select the Batch button again:



To export the table, choose the menu item **Output...**

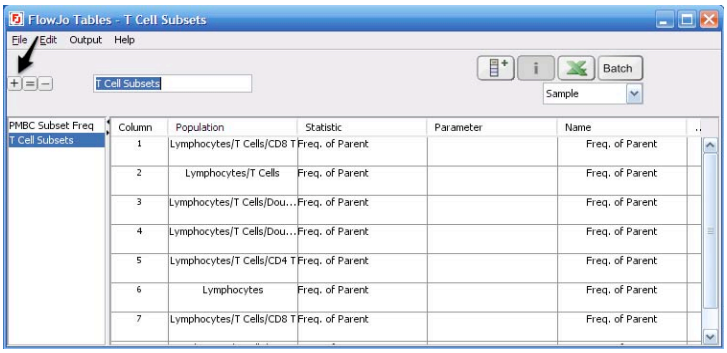


The length of the column headings often results in long names (as exemplified in the Excel spreadsheet below), often much wider than the space allowed for by the columns. However, you can keep the column names short by giving your own names in the final column of the Table Editor.

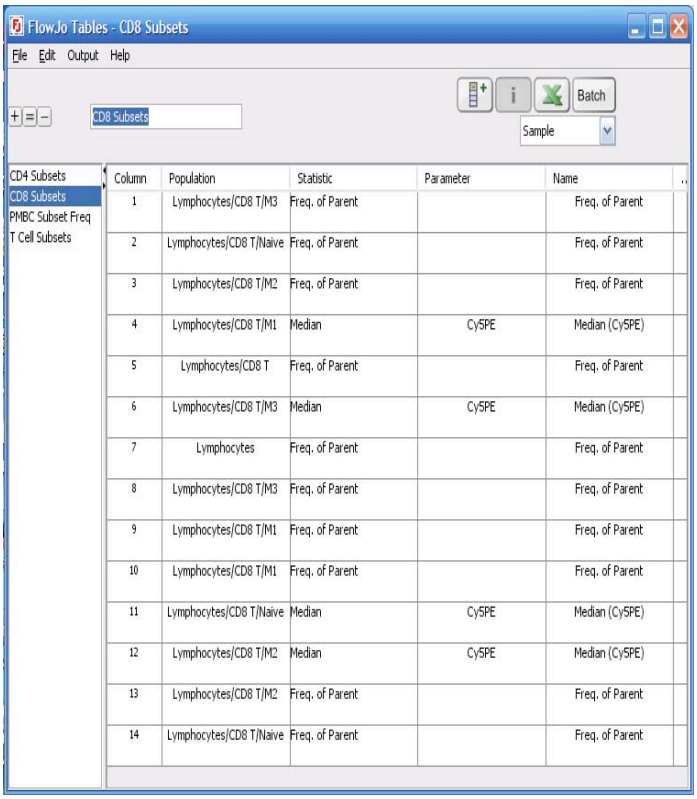


	A	B	C	D	E	F	G	H
1	Samples	Freq. of Parent	Freq. of Parent	Freq. of Grandparent	Freq. of Parent	Freq. of Parent	Freq. of Parent	Freq. of Parent
2	931115-B01- Sample 01.fcs	69.00%	91.17%	62.91%	91.17%	21.00%	47.00%	43.67%
3	931115-C01- Sample 02.fcs	77.57%	96.96%	75.21%	96.96%	14.58%	39.51%	47.46%
4	931115-D01- Sample 03.fcs	53.54%	92.87%	49.72%	92.87%	31.30%	54.06%	40.13%
5	931115-E01- Sample 04.fcs	54.68%	91.77%	50.18%	91.77%	33.37%	49.36%	45.07%
6	Mean	63.70%	93.19%	59.51%	93.19%	25.06%	47.48%	44.08%
7	Standard Deviation	11.62%	2.61%	12.12%	2.61%	8.84%	6.07%	3.06%
8								

The Table window is always current. When you create a table, FlowJo goes through all the samples and makes sure they are recalculated according to the latest modifications. If you now go back and change the lymphocyte gate, apply that change to all of the samples, then that change will be immediately reflected in the table window. From the Table Editor, you can create new table templates, duplicate existing tables, or delete existing table templates using the buttons across the top.


FlowJo Tables - T Cell Subsets
File Edit Output Help
T Cell Subsets
Batch
Sample
PMBC Subset Freq
T Cell Subsets
Column Population Statistic Parameter Name
1 Lymphocytes/T Cells/CD8 T Freq. of Parent Freq. of Parent
2 Lymphocytes/T Cells Freq. of Parent Freq. of Parent
3 Lymphocytes/T Cells/Dou... Freq. of Parent Freq. of Parent
4 Lymphocytes/T Cells/Dou... Freq. of Parent Freq. of Parent
5 Lymphocytes/T Cells/CD4 T Freq. of Parent Freq. of Parent
6 Lymphocytes Freq. of Parent Freq. of Parent
7 Lymphocytes/T Cells/CD8 T Freq. of Parent Freq. of Parent

Click on the Plus button and name the new table "T Cell Subsets". From the Workspace, select the **T Cell Analysis** group. Select the gate tree and drag it from the first sample into the Table Definition window; you will see all 8 rows: Create two more tables for the CD4 Subsets and CD8 subsets. Again, Select the gate tree and drag it to bring everything into the table. Note that you can change the order of the table entries by clicking on any entry and dragging it to another place. You can also delete a table entry by selecting it and pressing the ***delete or '-'*** key. After creating the last table, the Table Editor window should look similar to the one shown below.


FlowJo Tables - CD8 Subsets
File Edit Output Help
CD8 Subsets
Batch
Sample
CD4 Subsets
CD8 Subsets
PMBC Subset Freq
T Cell Subsets
Column Population Statistic Parameter Name
1 Lymphocytes/CD8 T/M3 Freq. of Parent Freq. of Parent
2 Lymphocytes/CD8 T/Naive Freq. of Parent Freq. of Parent
3 Lymphocytes/CD8 T/M2 Freq. of Parent Freq. of Parent
4 Lymphocytes/CD8 T/M1 Median Cy5PE Median (Cy5PE)
5 Lymphocytes/CD8 T Freq. of Parent Freq. of Parent
6 Lymphocytes/CD8 T/M3 Median Cy5PE Median (Cy5PE)
7 Lymphocytes Freq. of Parent Freq. of Parent
8 Lymphocytes/CD8 T/M3 Freq. of Parent Freq. of Parent
9 Lymphocytes/CD8 T/M1 Freq. of Parent Freq. of Parent
10 Lymphocytes/CD8 T/M1 Freq. of Parent Freq. of Parent
11 Lymphocytes/CD8 T/Naive Median Cy5PE Median (Cy5PE)
12 Lymphocytes/CD8 T/M2 Median Cy5PE Median (Cy5PE)
13 Lymphocytes/CD8 T/M2 Freq. of Parent Freq. of Parent
14 Lymphocytes/CD8 T/Naive Freq. of Parent Freq. of Parent

Notice in that picture how the column names were assigned for each of the columns generated in the CD8 Subsets table. These names will appear both within FlowJo's tables, and when the data is exported to other applications.

You can now apply these table templates to the appropriate groups and get output tables with specific sets of statistics: just select any group and click on the Table button.

Remember, you can apply a table template to any group; this could be useful for separately analyzing different experiments (that you have grouped separately). If you apply a table to a group that has no gates as requested by the table, you will get a lot of empty values.

Tables can also be added to Layouts in the ***Layout Editor***. Simply use the generated Table menu item **FlowJo > Add to Layout** in a generated table window and FlowJo will create a live table that updates as you create batch graphical layouts.

Table definitions are saved with the Workspace. When you re-open the Workspace, you can recalculate the tables. Tables can be applied to any group; you can, in the future, read more samples into the Workspace and apply the table to those samples. Table templates are a useful "batch analysis" tool; they allow you to quickly collate many statistics from many different samples for analysis by other programs.

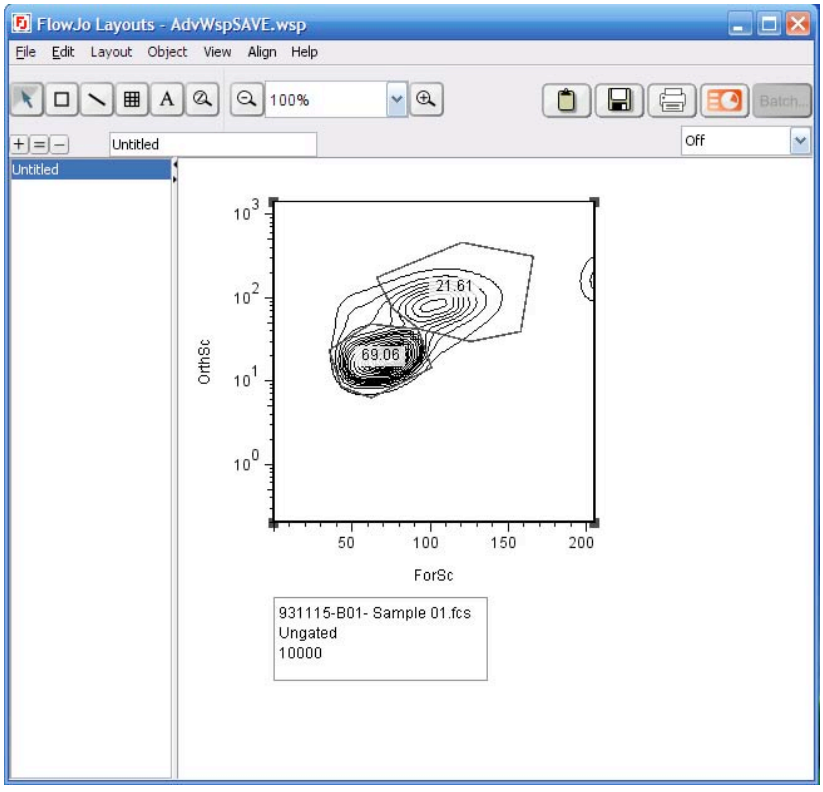
Lesson 7: Creating Simple Graphical Layouts

In this chapter, you will learn the fundamentals of the Layout Editor. You will be able to generate layouts with multiple different graphics, text items, lines, etc., and you will learn how to create overlays of graphs. In Chapter 8, you will learn how to create Batch Reports, where the layout is applied to all of the samples in the workspace. Finally, in Chapter 9, you will learn a few of the features of the Layout Editor that let you create complex multi-sample layouts, including statistics and graphs from multiple tubes in a panel.

This lesson continues with the Workspace document you have finished from Chapter 6; alternatively, you can open the workspace named "Tutorial WS (Chapter 7)".

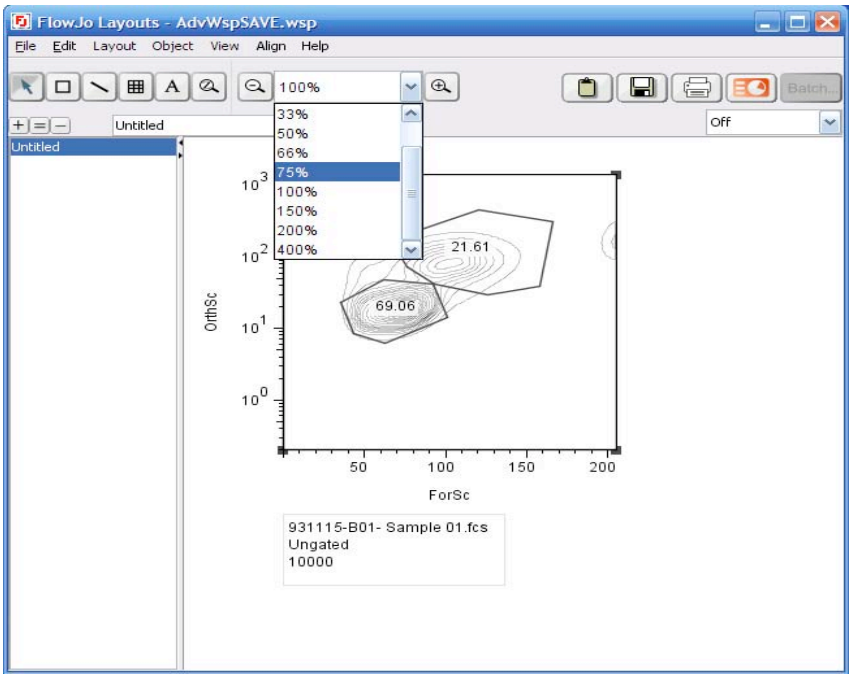
To open the Layout Editor either select **Layout Editor** from the **Windows** menu, press **Ctrl-L**, or click on the Layout icon in the row of icons at the top left of the Workspace window. You will be shown an empty layout editor.

In the ***Layout Editor*** window, the left portion of the window is a list of all of the layouts you have created for this workspace. You can have as many layouts as you wish. (For example, in the Demonstration Workspace shown in the introductory chapter, there were Layouts for generating patient reports, for verifying scatter gates, etc). You can name the layout by clicking in the name field (**Untitled-1**) near the top of the window and typing a new name.

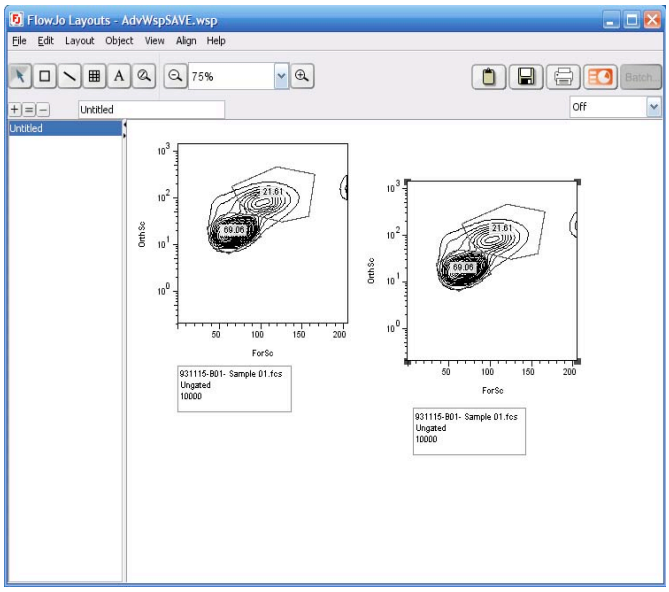


To add a plot to the Layout, click on any subset in the workspace and drag it into the layout view. For now, click on the top-level (ungated sample) "**931115-B01...**" and drag it into the Layout View. You will immediately see a graphic corresponding to the subset. (The default graph that is shown is the same as how the subset was last viewed). In addition, FlowJo creates an Annotation text box below the graphic that contains some pertinent information. To view gates, double-click on the graph (this brings of up the Graph Definitions dialog box) select the Annotate tab, click in the "Show Gates" box (you should see a check mark when selected) and click ok at the bottom of the dialog box.

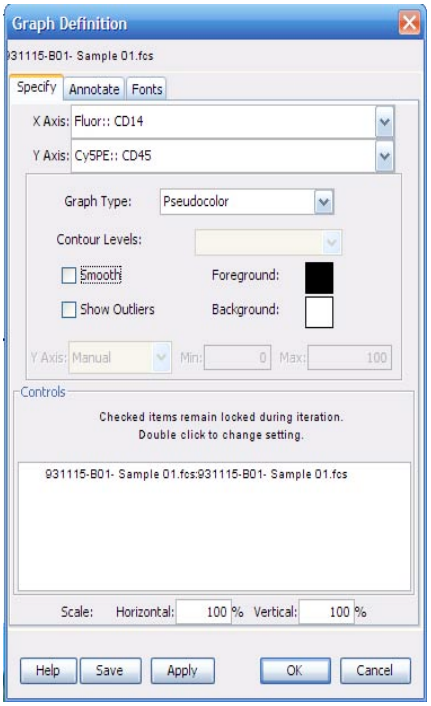
Any graphic item can be resized or moved just by clicking and dragging. To resize, click and drag on one of the four handles at each corner. You can also change the magnification of the view by clicking on the Scale popup menu near the top of the window (see example, right).



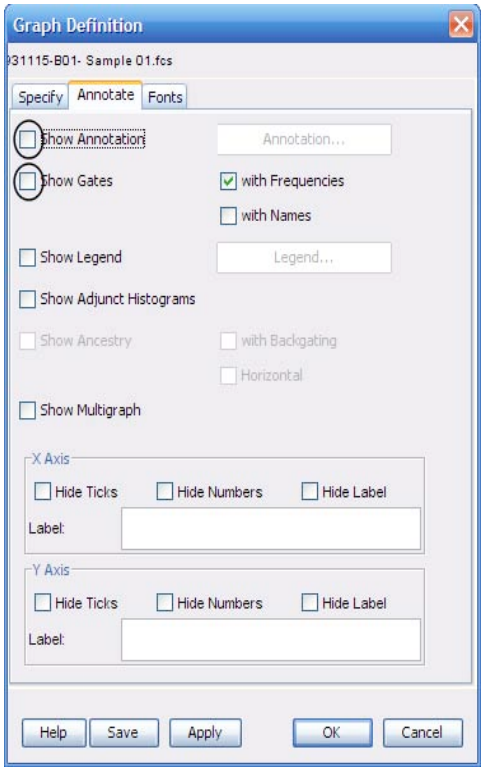
One of the important aspects of the ***Layout Editor*** is that it is live. This means that any time you change or move a gate or modify an analysis in the ***Graph*** Window, FlowJo will automatically update the Layout Editor if needed. Thus, you can use the Layout Editor to provide instantaneous feedback for gating operations, where you can simultaneously view many different subsets (or even multiple views of the same subset) while moving a gate used to define that subset.



To create another view of the same subset, you have four choices: (1) drag the same subset from the Workspace window into the Layout Editor again; (2) select the first graph in the Layout Editor, and do a **Copy** and **Paste** operation; (3) right click and select **Duplicate**; or (4) Hold the Alt key while clicking the graph. For now, ***duplicate*** first graph and move it over to the side). You will note that the second graphic is identical to the first.

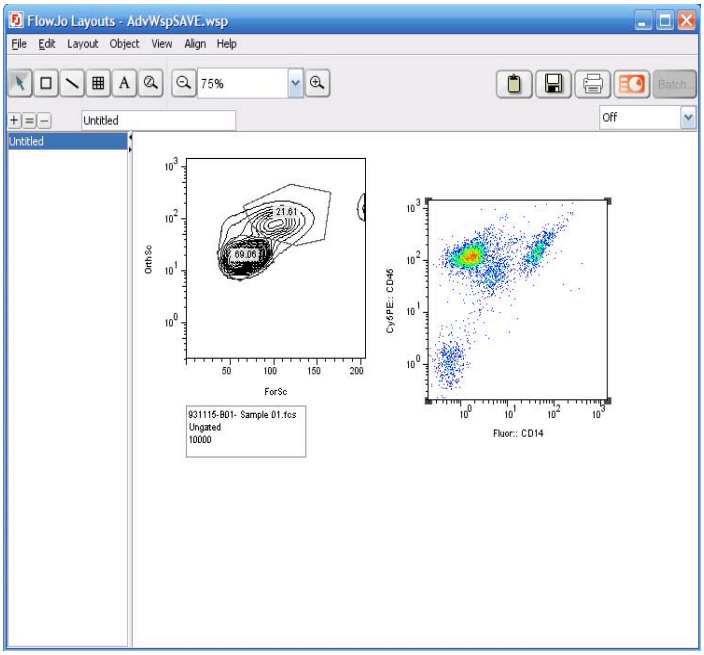


To change how it looks, double click on the graphic; FlowJo shows you the ***Graph Definition*** dialog as shown left. From this window, you can specify exactly how you want the graphic to appear. As shown in the example, change the X and Y axes to **CD14** and **CD45**, and change the graph type to **Pseudo-color** and uncheck smoothing.



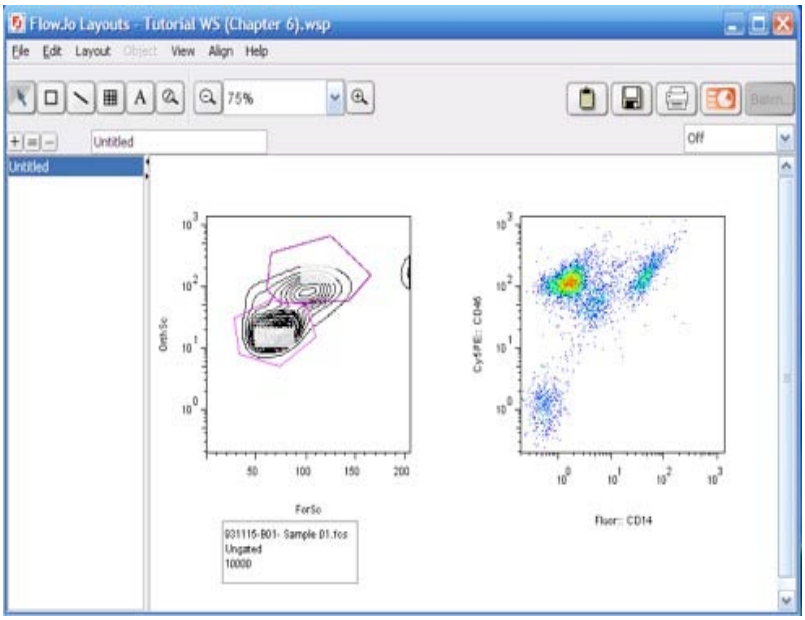
Now click on the Annotate tab near the top of the window. This shows a different set of options that control what appears in the graphic (see right).

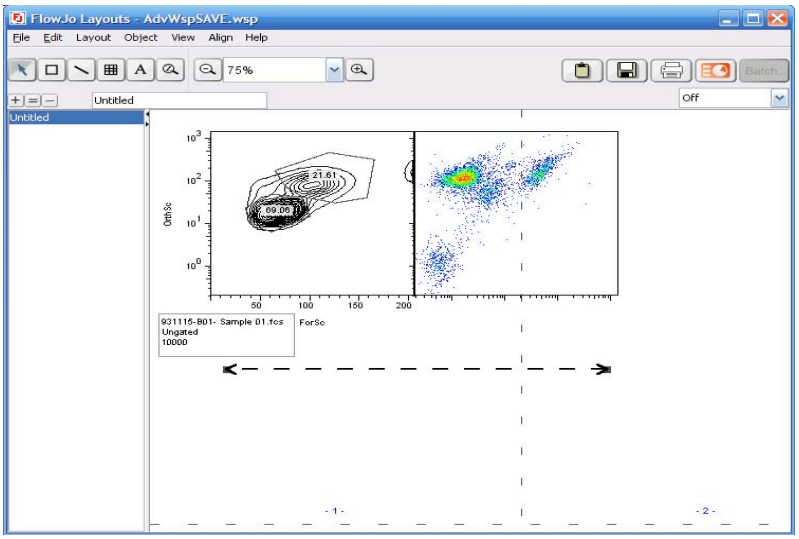
Unselect the checkbox **Show Annotation** and **Show Gates**: this will remove the gates and the annotation text below the graphic. Click on **OK**, and the ***Layout Editor*** should appear as follows:



FlowJo provides many features of drawing programs, such as the ability to align multiple objects. To align the two graphs, select them both (use a marquee selection tool by clicking and dragging to encompass both graphs), and then choose the **Align** menu. You may choose to **Align** or **Distribute** objects in either dimension. For now, choose **Tops.** The ***Layout Editor*** will reflect your changes as shown on the next page.

Sometimes, adjacent graphs have exactly the same Y axis (or X axis). FlowJo lets you remove the axis labels to align the objects to be adjacent to each other – easily creating compressed graphical presentations suitable for publication. Double-click on the rightmost graphic, and, under the Annotate options, choose to hide the Y-axis numbers and labels and the X-axis numbers and labels.



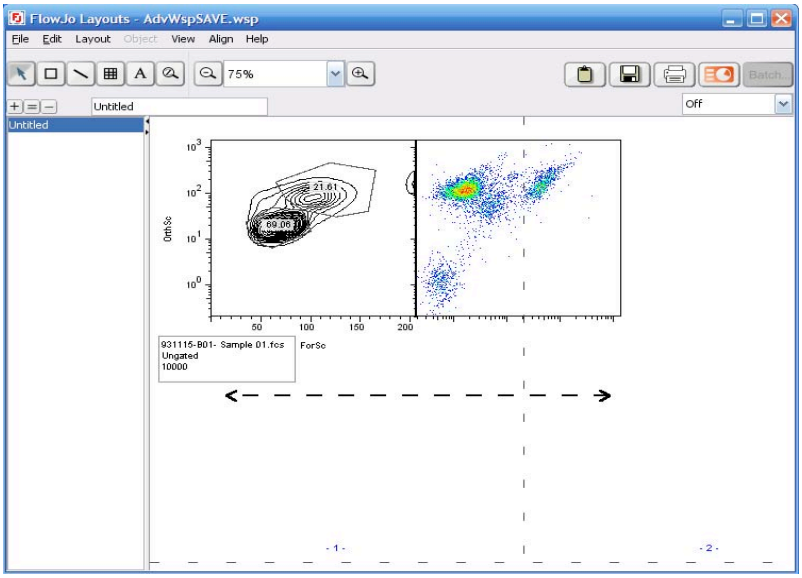


Now you can move the right graphic in the ***Layout Editor*** side-by-side, sharing a common Y axis. (Of course, in this example, the Y axis is different for the two plots; however, FlowJo still lets you put them side by- side. The layout will now appear as shown at left.

FlowJo's ***Layout Editor*** provides a few simple drawing tools to elaborate your graphical reports. These tools can be selected by clicking on one of the icons on the upper left edge of the window (as shown below). You can choose a ***Rectangle*** tool (to draw filled or unfilled rectangles) a ***Line*** tool (to draw lines and arrows) a ***Grid*** tool (to create grid-shaped containers), a Text tool (to create text objects), or a ***Zoom*** tool (zoom in on a specific graphic). Click on the ***Line*** tool, and draw a line by clicking and dragging underneath the two graphics. If you now double-click on the line (or, right-click the line and choose **Properties**... from the pop-up menu you can change the style of the line.

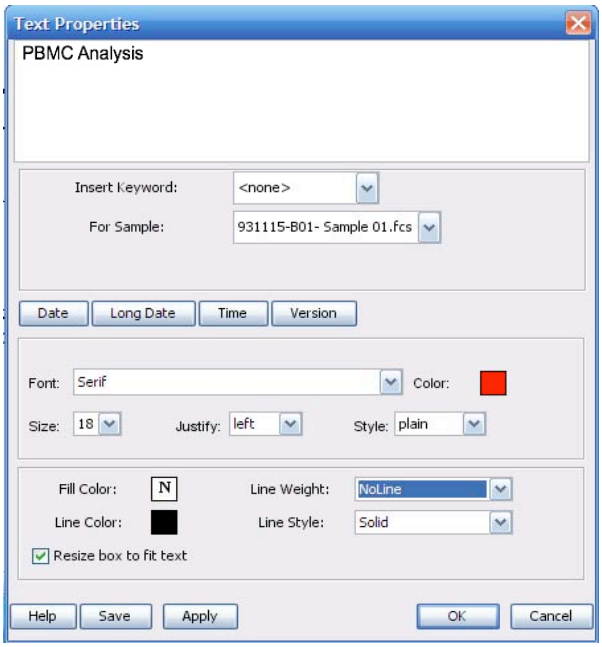
FlowJo shows you the ***Object Properties*** window as shown to the right.

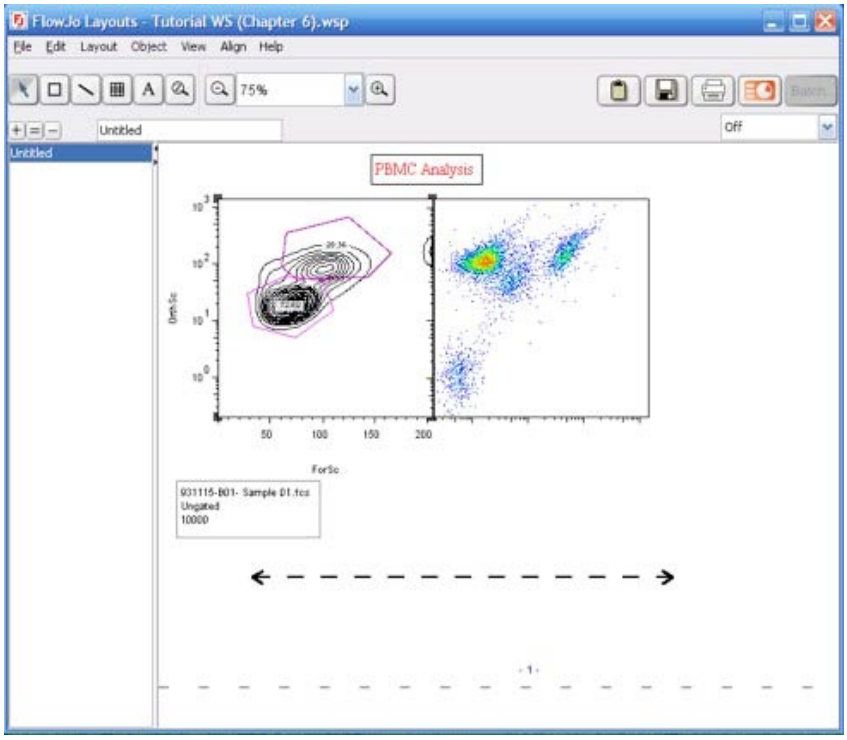
Change the line to be dashed by selecting **Dashed** in the **Line Style** popup menu; and select the line to have arrows at both ends by choosing **Double-headed** in the **Arrow Style** popup menu. If you wanted, you could also change the Line Color for the line. Click on the button marked **OK.** The dialog box will go away. Your ***Layout Editor*** should now show the dashed arrow, and look something like the one shown here:



To create a text object, click on the Text tool, and then click anywhere in the Layout View. (To create a text box with defined boundaries, click and drag the boundaries you want to have). FlowJo displays the **Text Properties** window, into which you can type any text. (You can also drag statistics from the Workspace and add keyword values by clicking on the **Insert Keyword** menu.

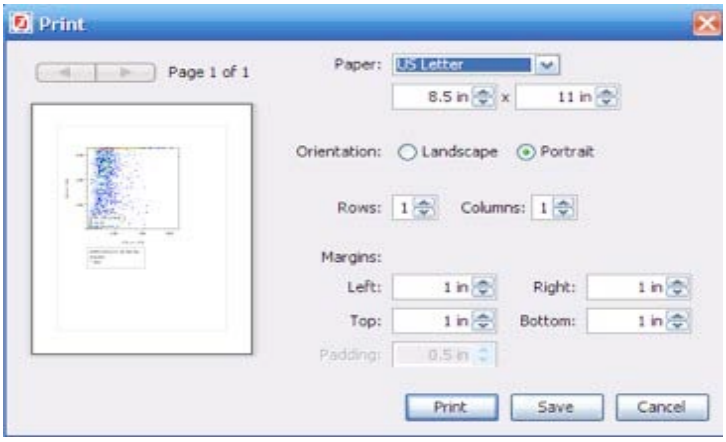
These items are live and will change as you create batch layouts for multiple samples!). Type "PBMC Analysis" into the window. You can also change the font and style of the text in the **Text Properties** window. Change the text color and font size as shown at right. Select the text **Color** to be red, and the size to be 18. (Note: the fill color is applied to the whole area of the text box; the Line color is applied to the box drawn around the text box – which is drawn only if the **Line Weight** is set to something other than **None**). Click on **OK** to confirm the changes. Your ***Layout Editor*** should appear as the example on the next page.





Layout Graphics can be easily exported, saved, or printed. To copy a subset of the items in the layout, select the ones you want and choose Copy; now you can Paste into any drawing program. You can export the entire contents of the Layout Editor window by selecting from the menu File > Export to App…

In Edit > Preferences > File Formats, you can choose between different formats in which to export the contents: gif, jpeg, pdf, png, svg or emf.

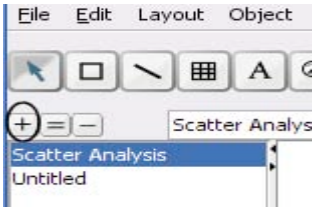


With **File > Print…** in the ***Layout Editor*** you can change the Page Setup**...** to orient the page and change the paper size and page margins to your needs. The dashed gray lines drawn in the ***Layout View*** represent the edge of the printable area; to view these lines,

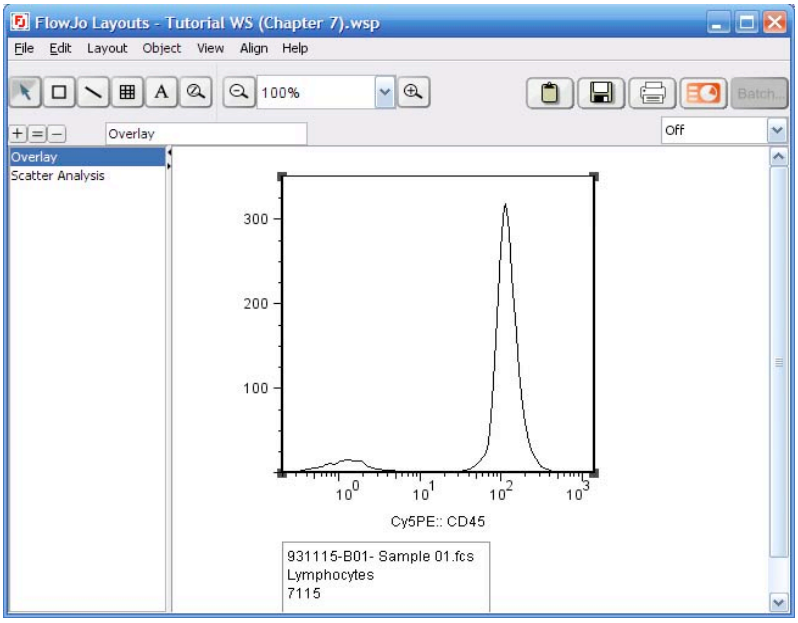
click View from Layout menu bar and choose "Show Page Breaks", you can drag these at any intersection to adjust the print area per page of your layout. Page margins are added outside these lines when printing.

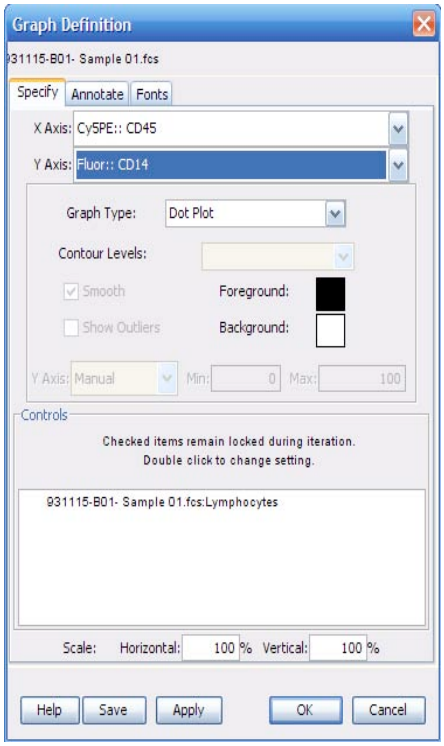
You can also copy items from layout to layout (within the same workspace. Click on the first graphic only, and choose **Copy** from the **Edit** menu (or press Ctrl+C). You can now create a new layout and paste that object into the new layout.

As noted before, you can have as many different layouts in each Workspace as you wish. To create a new layout, click on the **+** button near the top left of the window. (To delete a layout, click on the **-** button).



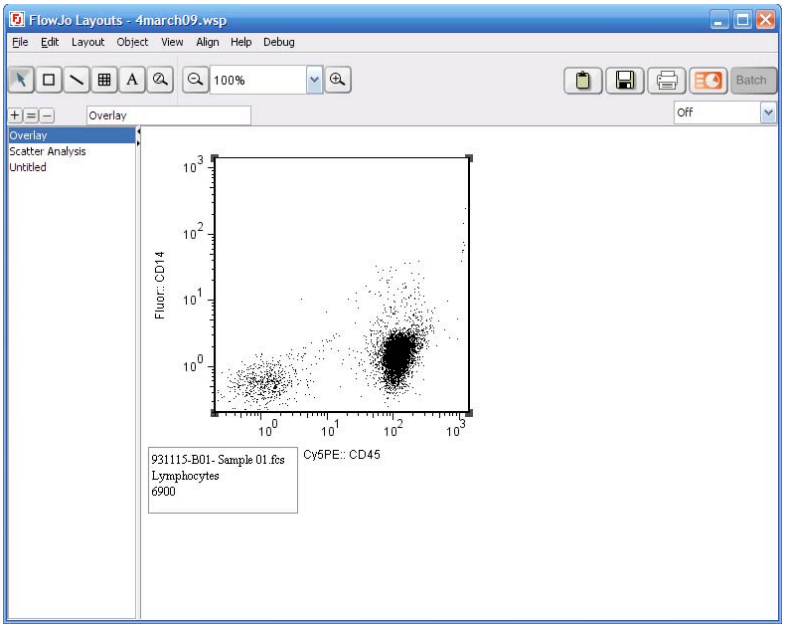
Now, let's create a second layout to demonstrate how to create graph overlays. Create a new Layout and name it "Overlay". Drag the first **Lymphocytes** subset (from **9 3 1115-B01..).** and drop it in the right hand pane of the ***Layout*** window. You should have a view of the CD45 histogram as shown here:



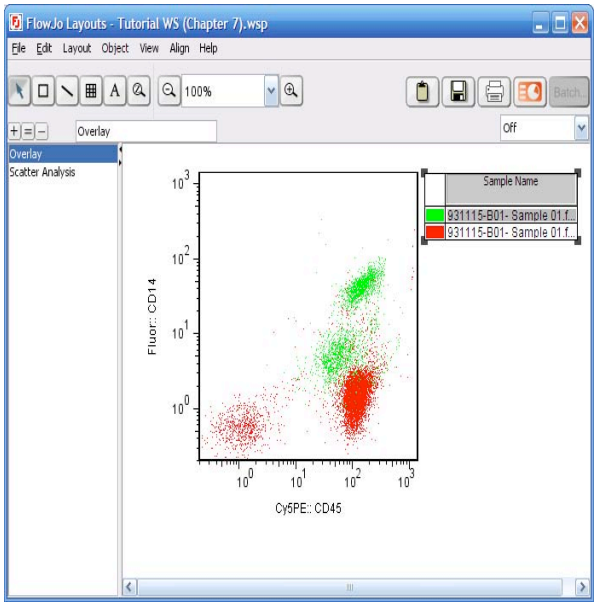


Double-click on the new plot. The **Graph Definition** window will appear as shown left. Change the graph specification to be **Dot Plot** of **CD45 vs. CD14**.

Click on **OK**, and the Layout View now displays a dot plot, as shown below.

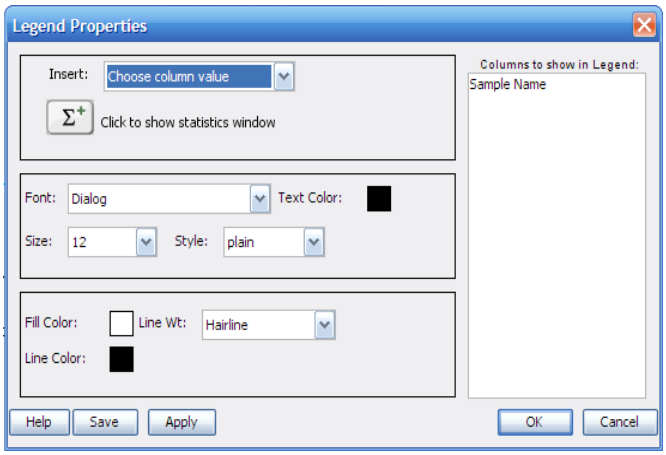


Any graphic item can be made into an overlay by dragging any other subset and dropping it onto the overlay.



You can overlay different subsets from the same sample, or overlay plots from different samples. For now, select the **Monocytes** subset from the same sample and drop it on the graphic (the sample name will appear in the legend to show that is was dropped). You should now see the multi-color dot plot shown left.

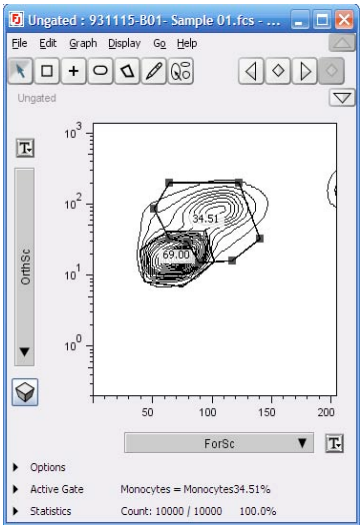
FlowJo draws two dot plots, one for each subset, in the same graph. In addition, it automatically creates a ***Legend*** for the overlay, shown to the right of the graphic. You can change the Legend properties by double-clicking on the legend. Add the Population Name by choosing it from the **Insert** popup menu and remove the Sample Name by clicking on it and pressing the delete key. If you click on **Save** this settings will be applied to your preferences. Click **OK**.

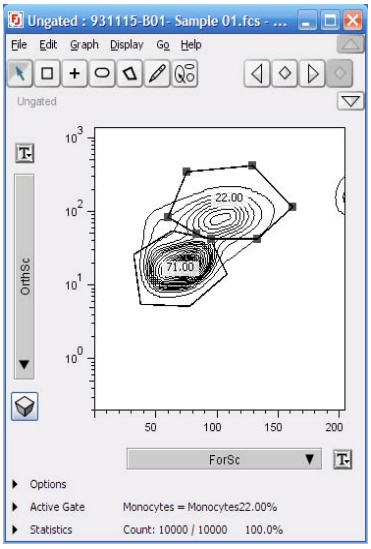


You can change the color of any subset by clicking on the color box next to that subset in the ***Legend*** text box. You can change the stacking order of the colored layers by clicking on any item, and dragging it up or down in the list. Finally, you can delete an item from the overlay by clicking with the right mouse key on the subset and choosing **Remove layer**. You can overlay an almost unlimited number of different subsets on the same graph.

Note: you can choose to show the ***Legend*** box for any graphic, even if it is not an overlay, from the **Annotate** preferences in the **Graph Definition Window**. There you can also set color and line styles for single graphs just as you can for overlay graphs.

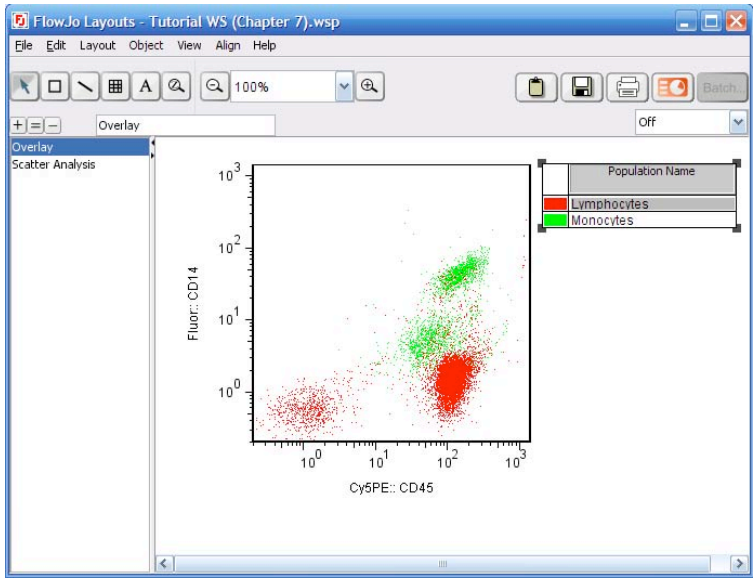
As noted before, all Layout graphs are live. This means that if you change a parent gate for one of the subsets, the graph is automatically updated. To see this in action, switch your attention back to the ***workspace***, and double click on the **931115-B01...** sample node. You should see the graph to the right, defining the gates for the **Lymphocytes** and **Monocytes** subsets: Click on the **Monocytes** gate (upper gate), and move it around – perhaps, as shown on the left, to overlap with the **Lymphocytes** subset.

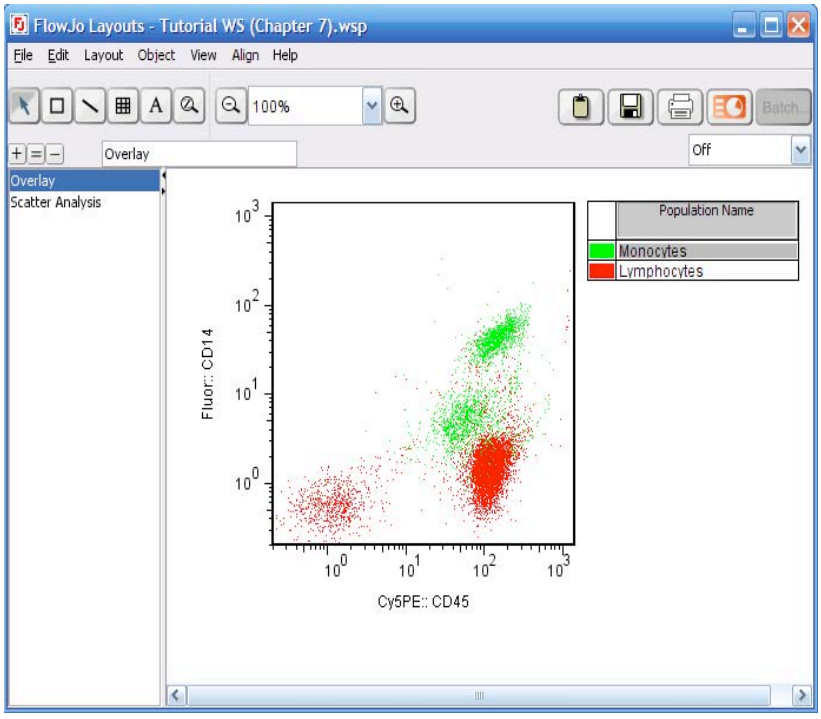




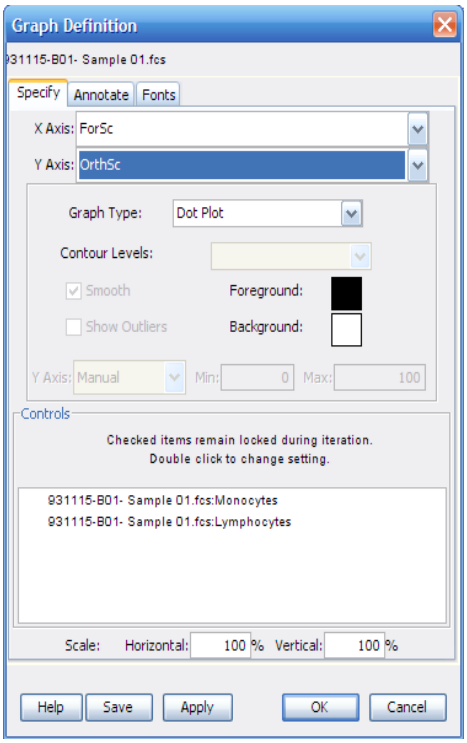
Note that the Layout Editor responds by updating the green dots (corresponding to **Monocytes**; see below). The order in which the subsets are drawn can have a significant effect on what the graphs look like. To change the order, click on any subset name in the legend, and drag it up or down (the cursor changes shape to let you know what is happening).

For example, click on the **Lymphocytes** subset, and move it to the top. Note that the area which has both green and red dots now appears to be completely red, because the red population is on top (i.e., drawn last) -- see the example below.





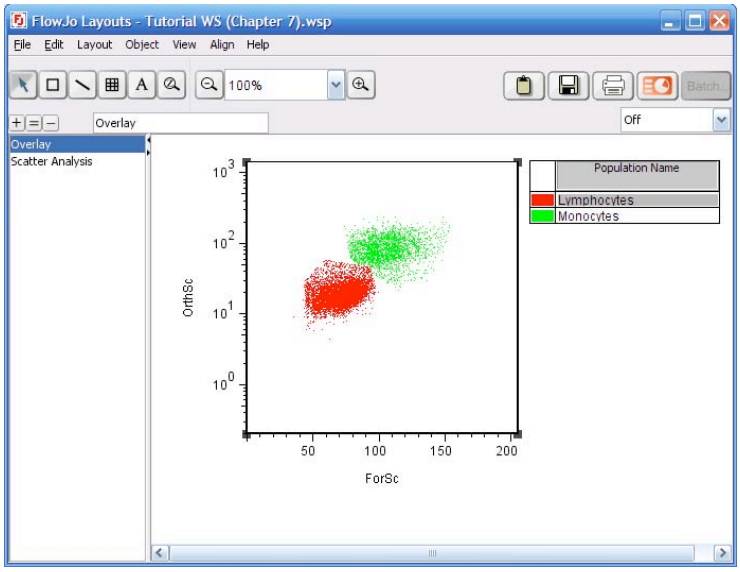
Once you create an overlay graphic, you can easily change its appearance (axes and plot style) – just like any other graphic.

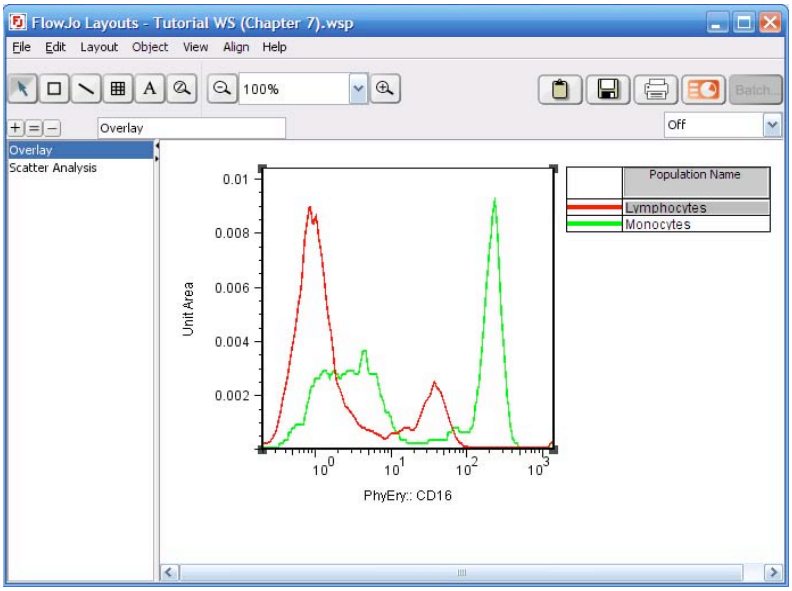


Double-click on the item; you will be shown the **Graph Definition** window. Change your graph to show **ForSc vs. OrthSc** (forward vs. side scatter).

Now, the ***Layout Editor*** Window should look something like the image below. See that the dot clusters reflect the gates which defined their colors.

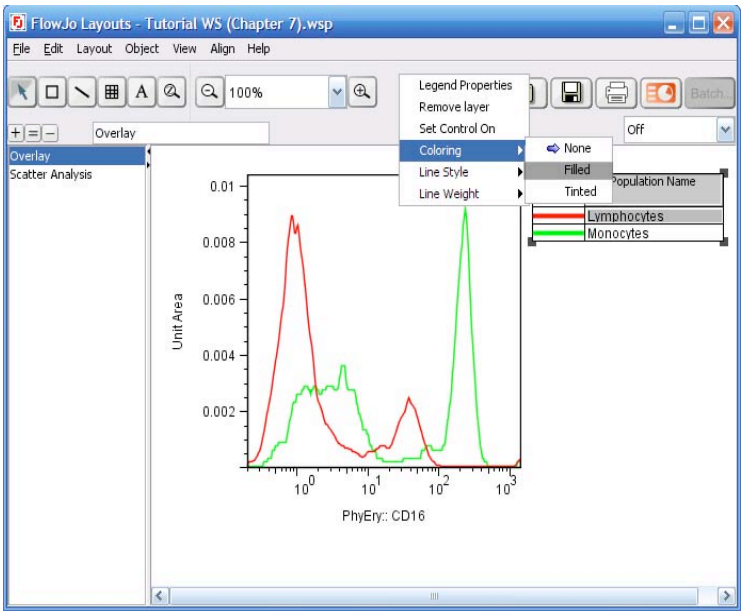
Double click on the graphic again, and choose to display a histogram of CD16. FlowJo now shows you a histogram overlay, like that shown below.





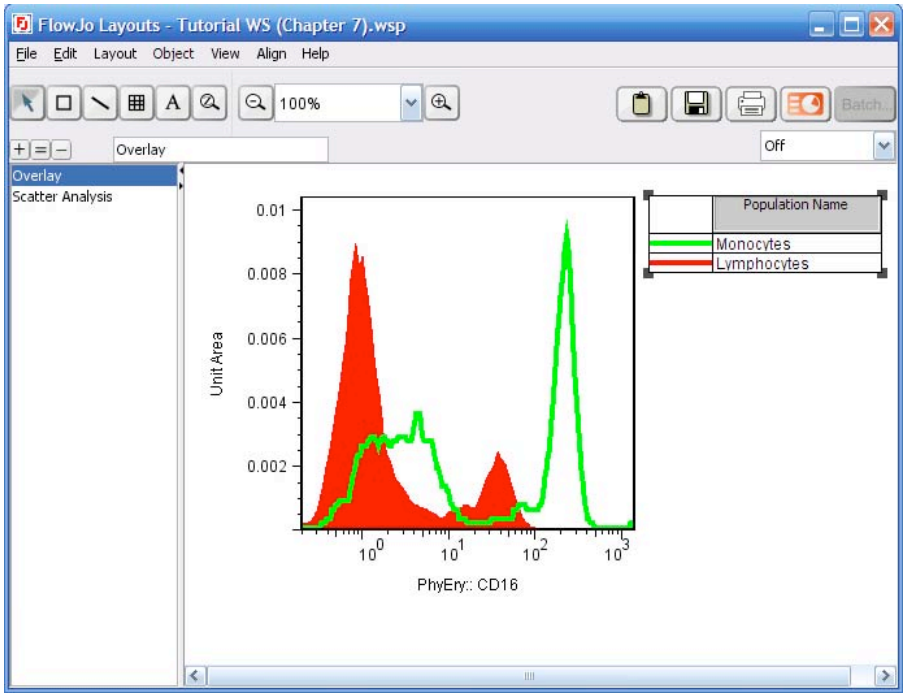
If you right click on the legend you will note that the popup menu now has an additional set of menu items.

These are controls that let you set the histogram line and fill style for each subset. If you right click on the **Lymphocytes** name, in the legend, you will see a popup menu as shown in the graphic on the image below.

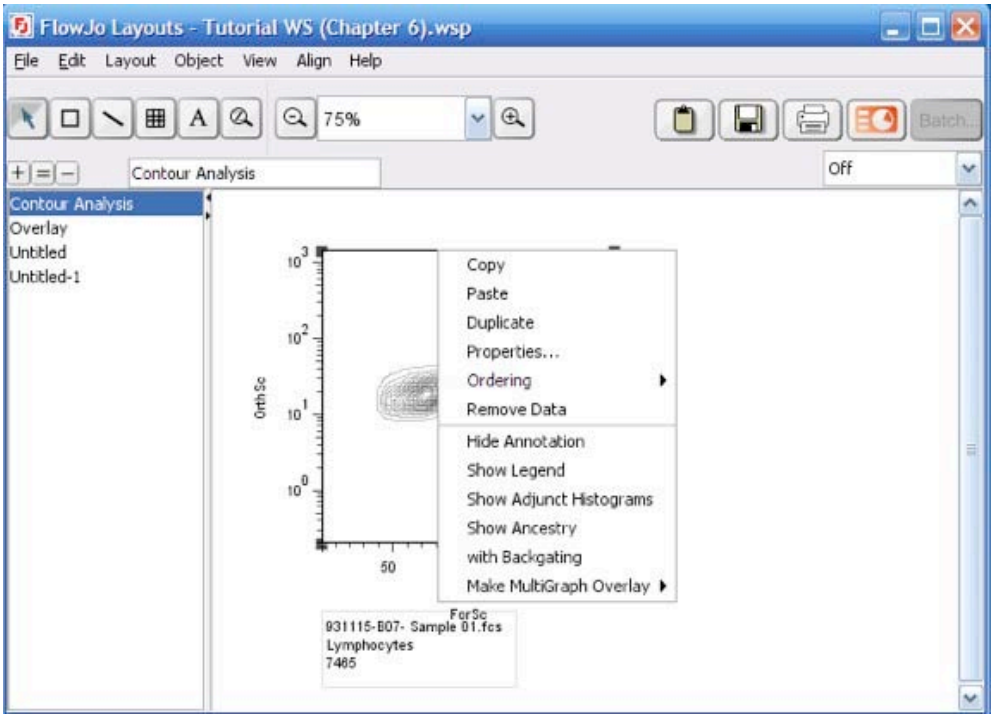


Here you can select the line weights (**Hairline, Normal, Heavy, or Thick)** the line styles **(Solid, Dotted, Dashed, Long Dashed, or Complex)** or if the histogram should be filled (**None, Filled** or **Tinted)**. Select **Filled**. Then right click on the line next to the green Monocytes color box, and select **Thick**. This will edit the line weight and the fill color of the two lines in the graph.

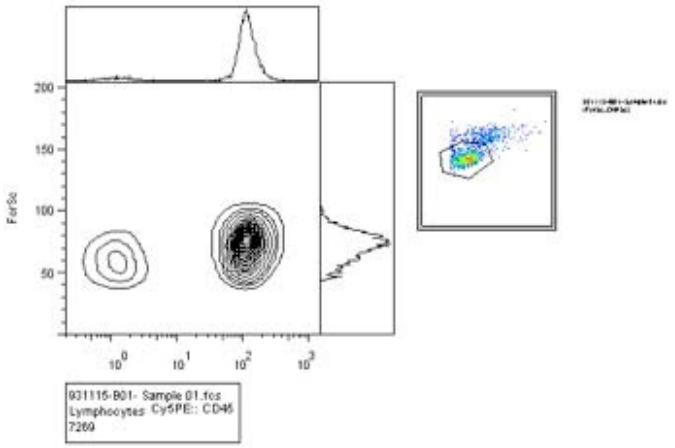
After dragging the **Monocytes** line to the first place of the legend your layout should show the overlay histogram as shown below.



Click on the **+** button and name the new Layout **"Contour Analysis".** Now select the Lymphocyte subset from the first sample, and drag it into the Layout Editor.



Because this plot is a subset, two additional options appear in the menu; **Show Ancestry** and **With Backgating**. Right-click the layout to see these options. With the first you can display subset plots showing the population's ancestry. FlowJo shows you not only the graph for a subset, but the plots of all parent populations as well, including their backgating if you so desire:



FlowJo gives you enormous flexibility in designing customized layouts, so that you can quickly and easily generate high-quality publication and presentation graphics.

Lesson 8: Creating Batch Graphical Reports

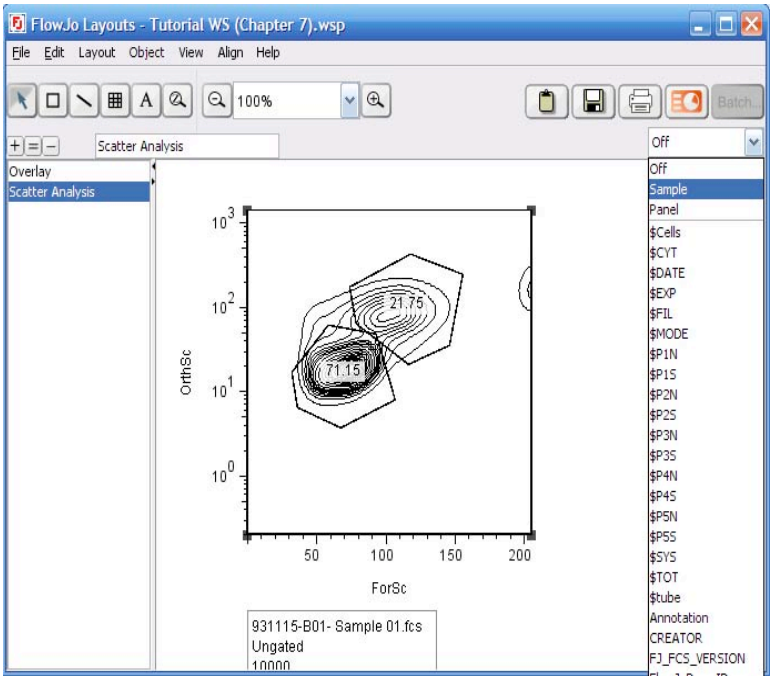
In this chapter, you will build on what you learned in Chapter 7 to generate graphical reports for entire experiments. You will learn how to cycle the Layout through different samples in the Workspace and how to create a combined output for printing or export of every graph for every sample.

This lesson builds on the Workspace you have finished from Chapter 7; alternatively, you can open the workspace named "Tutorial WS (Chapter 8)" and drag in the Experiment 1 data. Open the ***Layout Editor***, and select the "Scatter Analysis" Layout.

When you drag items into the ***Layout View***, FlowJo by default shows you the desired graph for the sample from which you dragged the subset. However, FlowJo can show you the corresponding graph from any sample in the current group. To do this, you must select an **Iteration Value** corresponding to the desired sample.

First, what is an ***Iteration Value***? In order to perform batch processing, FlowJo cycles over every sample in the current group (i.e., whichever group is selected and displayed in the workspace). Thus, in the **All Samples Group** for this experiment, there are 16 samples, and there are 16 different ***iteration values*** for the group: one for each sample. In the **CD4 Analysis** group, with four samples, there are only four ***iteration values***, corresponding to the four samples in that group. Normally, there is a one-to-one correspondence between an iteration value and a sample in the group. (In Chapter 9, you will learn how to iterate by other criteria to create more complex reports).

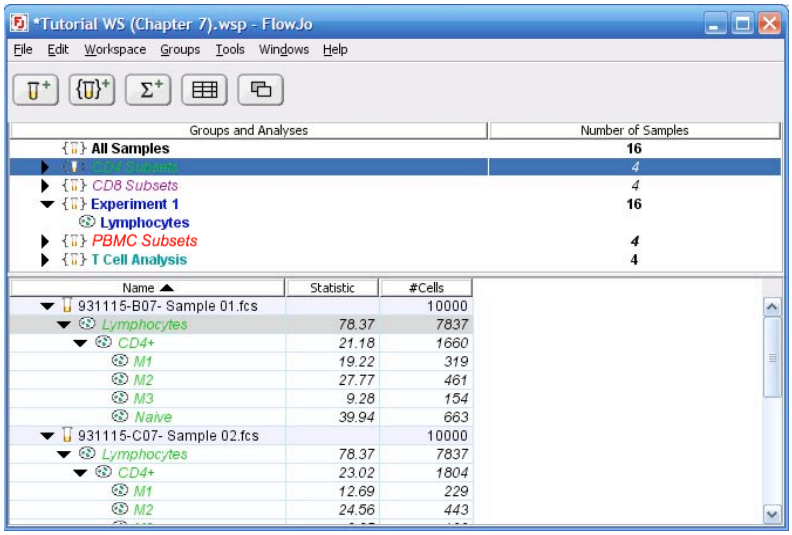
The Current ***iteration value*** is displayed and selected in the Iteration Popup menu, which is just below the Batch button at the right top of the ***Layout Editor.*** Whenever you create a new Layout, the current Iteration state for that view is set to **Off**. When Iteration is off, all of the graphs shown in the Layout are identical to the ones you dragged and dropped into the View originally. FlowJo doesn't care what the current group is. Select to iterate by Sample in the Iteration Type popup menu to enable the Batch button and to show the current ***iteration value.***

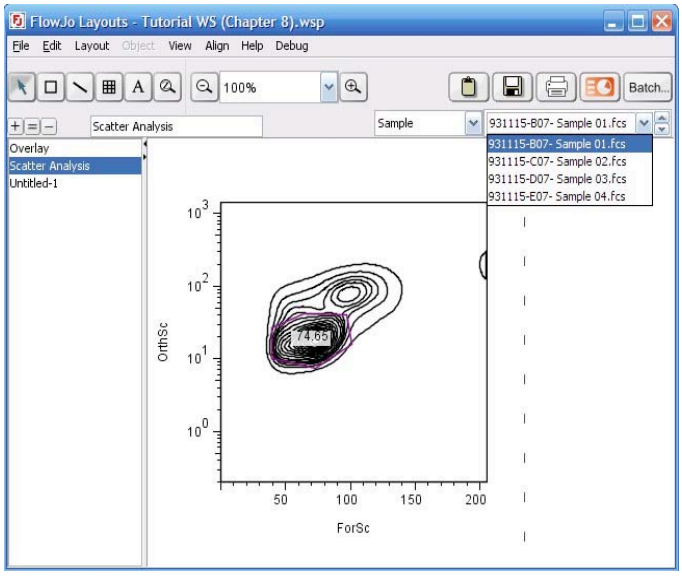


Because the current group is **All Samples**, FlowJo shows you the 16 different possible ***Iteration Values***—one for each sample in the current group. You can see all of these values in the Popup menu. Select one of the values from this popup menu (for example, the **931115-B01...**, as shown on the previous page).

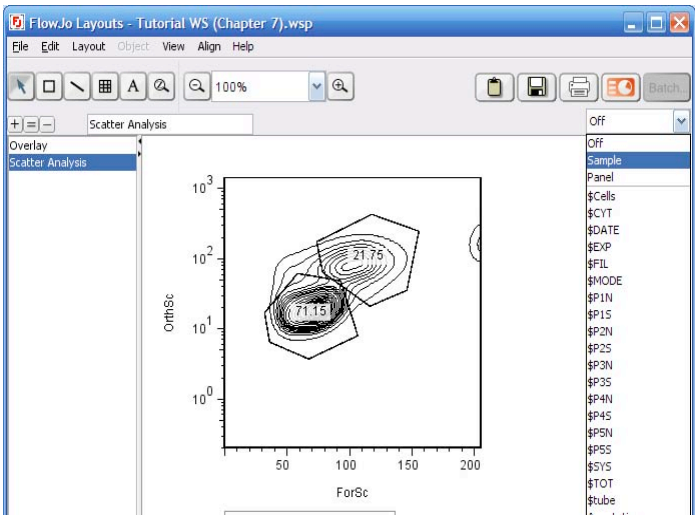
By selecting a specific ***Iteration Value***, you direct FlowJo to display the graphs in the layout view as they look for the sample you just selected. Therefore, the graphs you now see are those for the **931115-B01...** sample. (Note that if this sample did not have any of the subsets displayed in the Layout, then FlowJo would show an empty ***placeholder*** for the graph). Note the pair of arrow controls at the right edge of the window (right below the **Batch...** button). You can use these to increment or decrement the current iteration value by one - i.e., you can use them to cycle through successive samples in the current group.

Switch back to the Workspace, and select the ***CD4 Subsets*** group (see right). In this group, there are only four samples.





Now, if you select the iteration popup menu from the ***Layout Editor*** window, you will only be given a selection of four different samples to choose from (see left). Again, this is because whenever iteration is not off, FlowJo looks through the current group to decide what possible samples can be displayed.

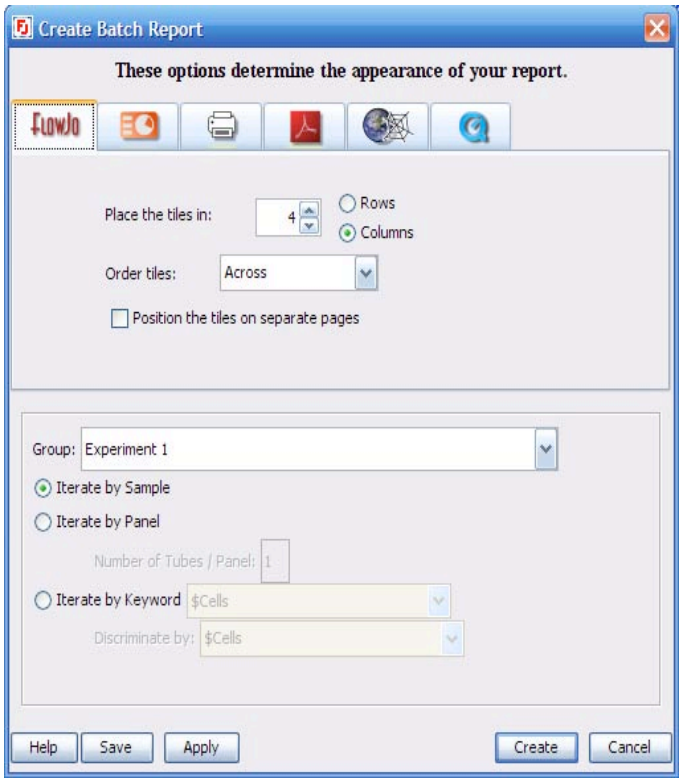


Note that if you construct a Layout for a specific group which has unique sets of gates and statistics, then the layout may not operate as desired on other groups which don't have those gates. Once again, if you select **Off** for the current iteration value, then FlowJo shows only the original graphs for this layout View (the ones that you dragged and dropped into the view), irrespective of what the current group is.

Understanding how FlowJo generates a graph for any given Layout item during batch processing is very important.

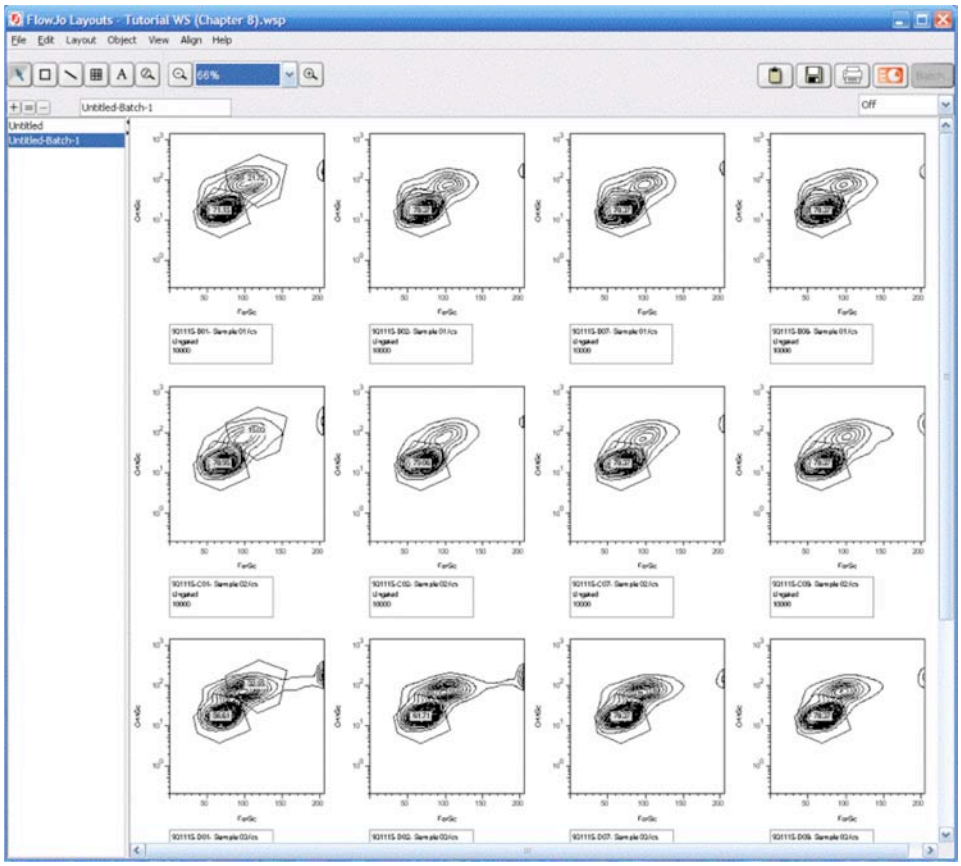
FlowJo draws a graph in the ***Layout Editor*** when all of the following criteria are met: (1) the current sample (i.e., the current value of the ***Iterator***) has the parameters that are displayed in the graph (like Forward Scatter, FL3, FL4, etc). and (2) the current sample has a gating tree that has exactly the same subset as what is desired in the graph (i.e., if the graph was dragged from a CD4 subset of Lymphocytes, then FlowJo looks for a CD4 subset of Lymphocytes in the current sample).

So far, you have only looked at the Layout Report for individual samples, one by one. To look at graphs for all samples at once, click on the ***Batch…*** button above the Iteration Popup menu. This dialog will let you set the options governing how the report will look. You can set the format of the report, the group that will be processed, the geometry of the rows and columns in the report, and other options to set FlowJo to perform the correct operation without asking you every time. Use the **Save** button to set these choices as the default. Select the **FlowJo tab**, set the tiles to 4 columns, set the Group to Experiment 1, select Iterate by Sample, and click on **Create**.



When you generate a Batch Output, FlowJo will start with the first ***iteration value***, and generate a ***page layout or tile*** for that particular sample. FlowJo then continues to generate a new tile for each successive Iteration Value until it has exhausted the current group. These tiles are used to create a New Layout window, such as that shown on the below.

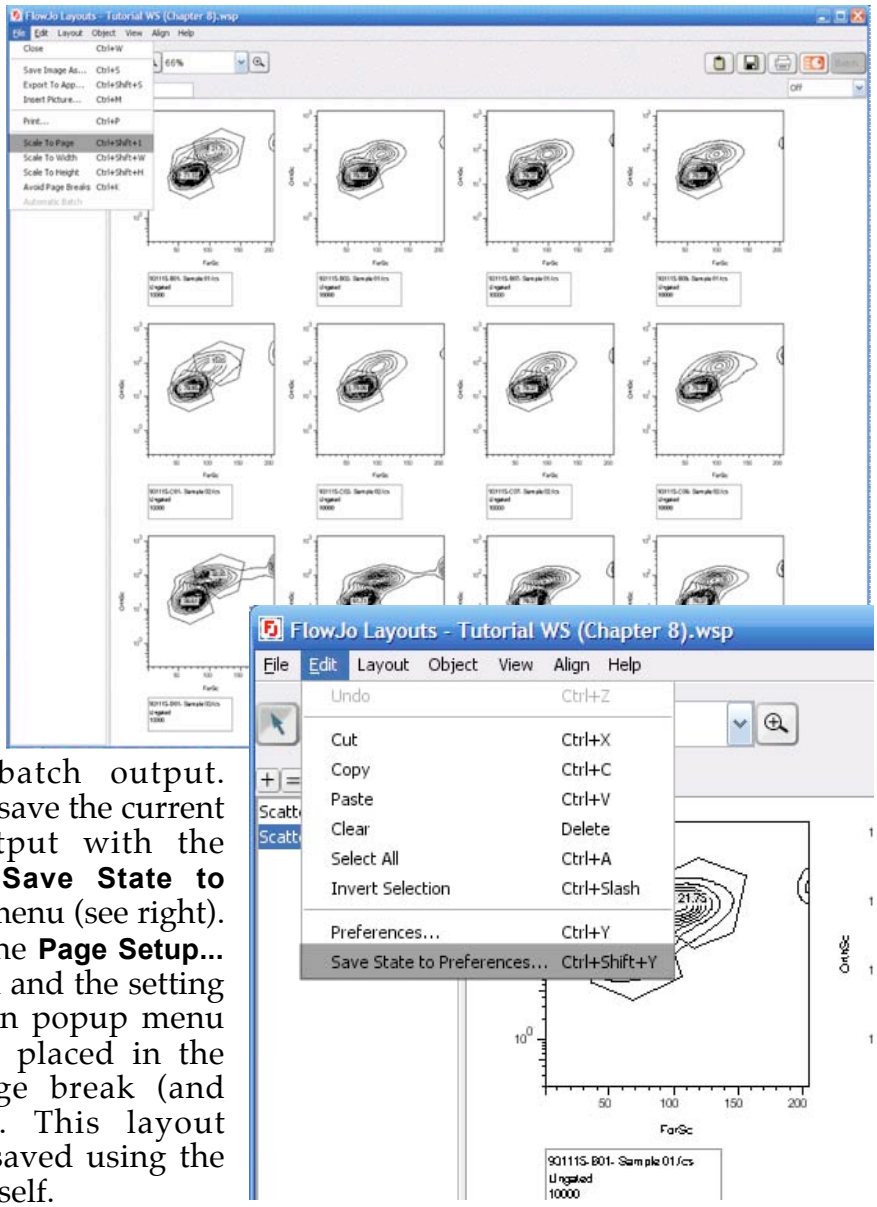
Batch Layouts can be generated in six different modes as shown as tabs. The first type will simply create a new layout in the layout editor with all of the iterated samples shown. This is the most flexible way to generate a report, as you can now edit the layout further, adding titles or annotations to specific graphs, or by removing graphs that are not interesting. The **PowerPoint Data** option generates a PowerPoint presentation in which each tile corresponds to a slide. You can ungroup these graphs in PowerPoint and edit them further. The third tab directs the output directly to the printer. You can choose how many sets of graphs (tiles) you want on each page. The forth tab will generate a PDF file with each tile represented on a new page. The fifth choice is **Write Web Page**, FlowJo creates a folder containing each tile as a separate graphic, and creates an ***index.html*** file to easily view the graphics from the World Wide Web. The last tab will create a **Quicktime Movie**, where each set of graphs becomes one frame of an animation. You can play the movie in any movie viewer and cycle through all of the graphics. All of the different views generated in these batch reports support printing, saving to disk, copying to the clipboard, and creating Web pages.



Now return to the new Layout view. There is a zoom control menu at the top of the window that adjusts the magnification of the view. You can scale to any percentage, such that the entire batch layout can be seen on the screen (see above).

You will notice that FlowJo draws gray dotted lines in the window: these correspond to page breaks in a printed document. (Select a small magnification, like 12.5%, to see many pages at once). Note that as you change the viewing magnification, the relative scaling of the graphs to the page boundaries do NOT change – you are not changing the print magnification!

Changing the orientation – landscape or portrait can be easily done using **Print** from the **File** menu and setting the paper type and orientation you want.
You can also specify that FlowJo automatically scale one page to be as wide as the graphic display. This is done by selecting **Scale To Page** in the **File** menu of the window (see right). If you select **Avoid Page Breaks**, from the same menu, FlowJo uses your paper type/shape, but places tiles on the page such that tiles don't cross page boundaries.

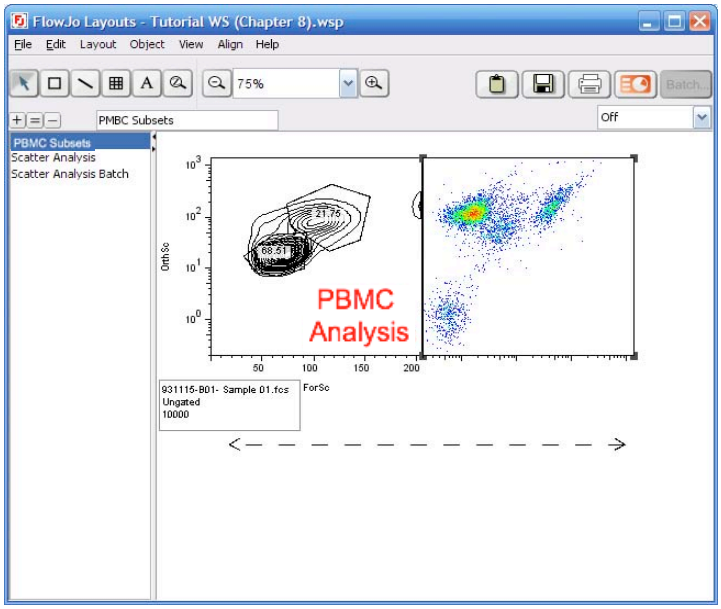


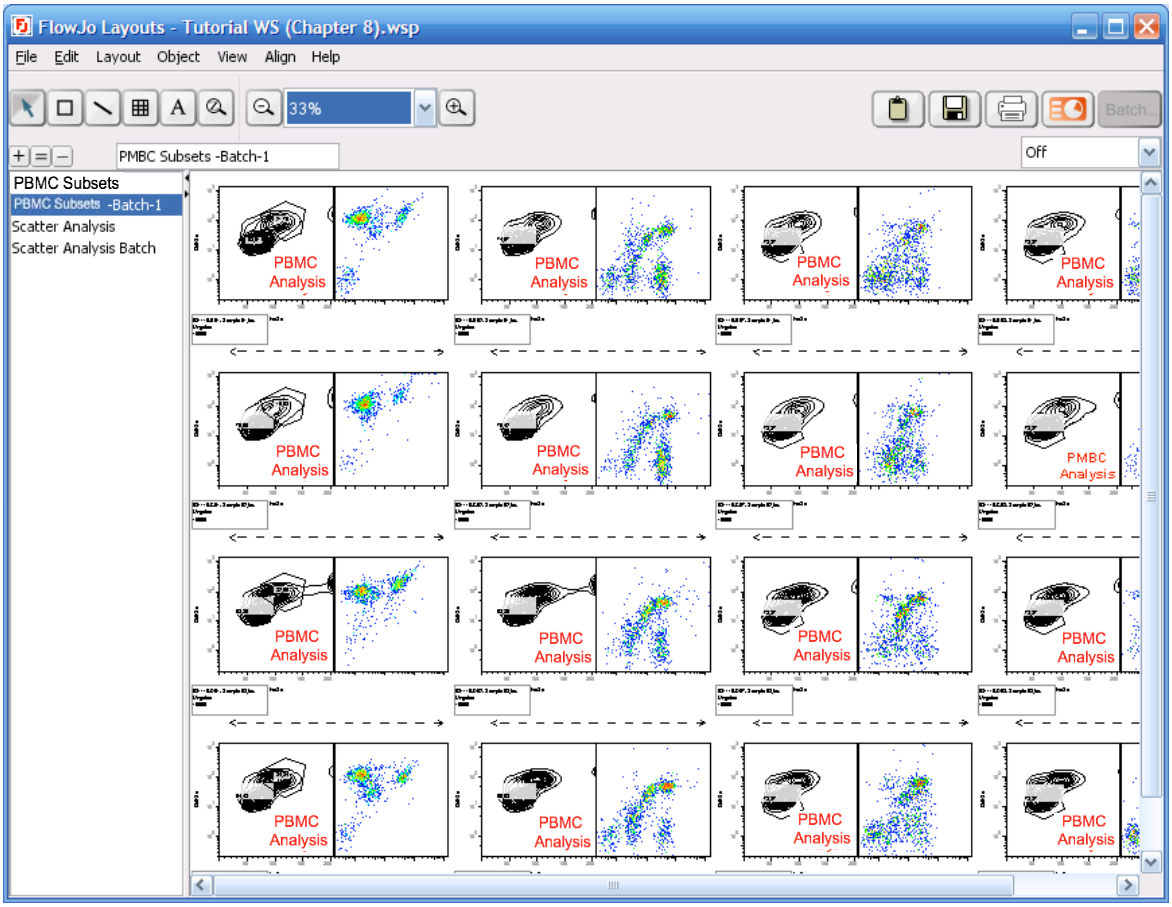
Once you have arranged the tiles exactly the way you want, you can print them – you will know exactly how they will appear on the pages.

It might be cumbersome to have to redesign the printing layout every time you create the batch output. Therefore, FlowJo lets you save the current state of the Batch Output with the Layout... simply select **Save State to Preferences** from the **Edit** menu (see right). This saves the values of the **Page Setup...** paper type and orientation and the setting of the Layout Specification popup menu (i.e., how tiles are to be placed in the overall output), the page break (and geometry) specification. This layout specification can also be saved using the **Save** button on the menu itself.

In the ***Layout Window***, select the original Layout (PBMC Analysis) that has the two graphics (as shown below).

These two graphics are a forward vs. side scatter plot, and a **FITC CD14 vs. Cy5PE CD45** plot. In the Batch Report screen, change the group to "Experiment 1". Select to Iterate by Sample in the Iteration Type menu to enable the Batch button. Click on the **Batch** creation button, and specify that you want to make a new layout (still four across). The new layout will be added to the list on the left-hand side. Its name is the original layout followed by the suffix -Batch. It should look something like that shown below called **Untitled-1 Batch**.



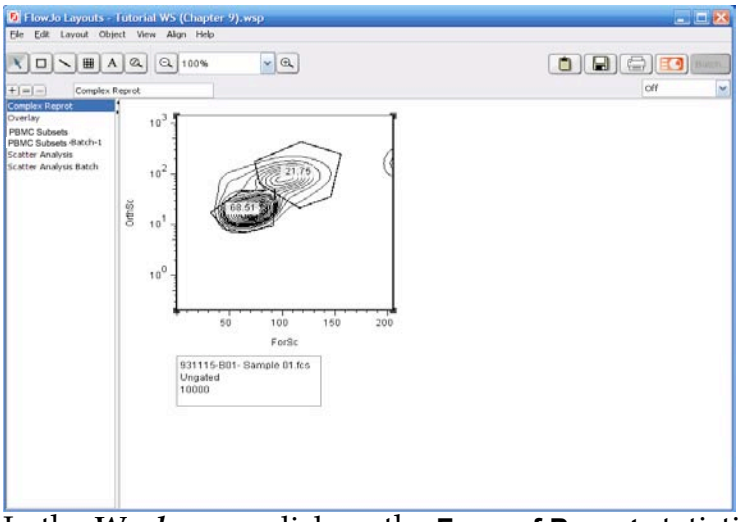


Now, each tile consists of two plots, the arrow line, and the text item. Remember that FlowJo creates a new tile for every sample in the current group.

One last important piece of information; the ***Layout Editor*** view is ***live*** in that whenever you change a gate or analysis that might affect the view, the view is automatically updated to reflect the change. However, the **Tiled Report, Web Report and Movie** are **NOT** live. If you create one of these reports and then change a gate, the report is **NOT UPDATED**. You will have to regenerate the ***batch output*** to reflect in these media the change you have made.

Lesson 9: Generating Complex Batch Analysis

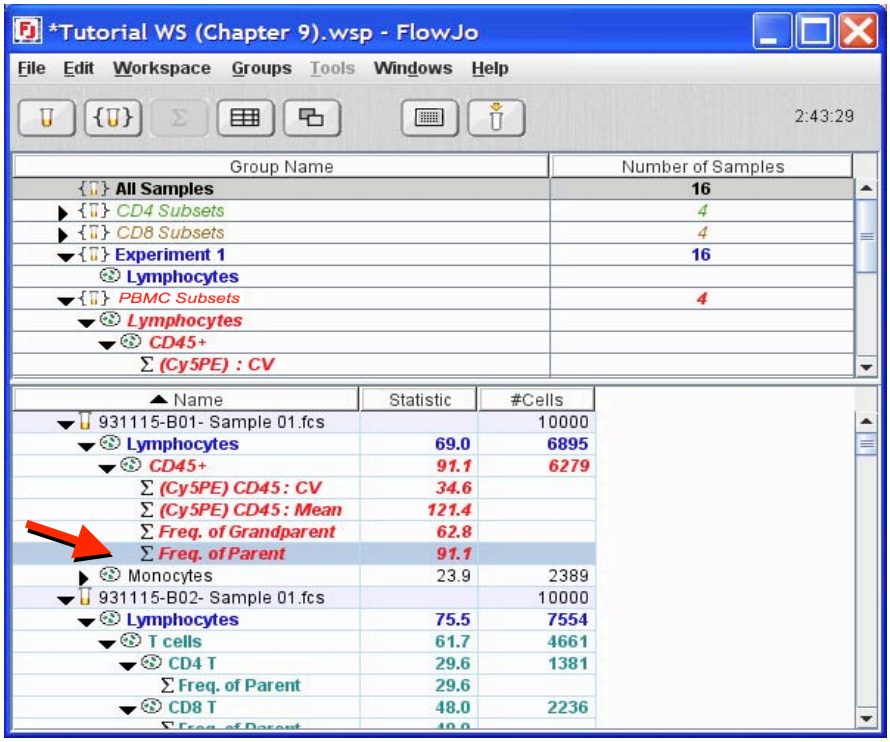
In this chapter, you will build on what you learned in Chapter 8 so that you can generate graphical reports that include statistics, as well as graphical reports that draw graphs from multiple different tubes for each tile.

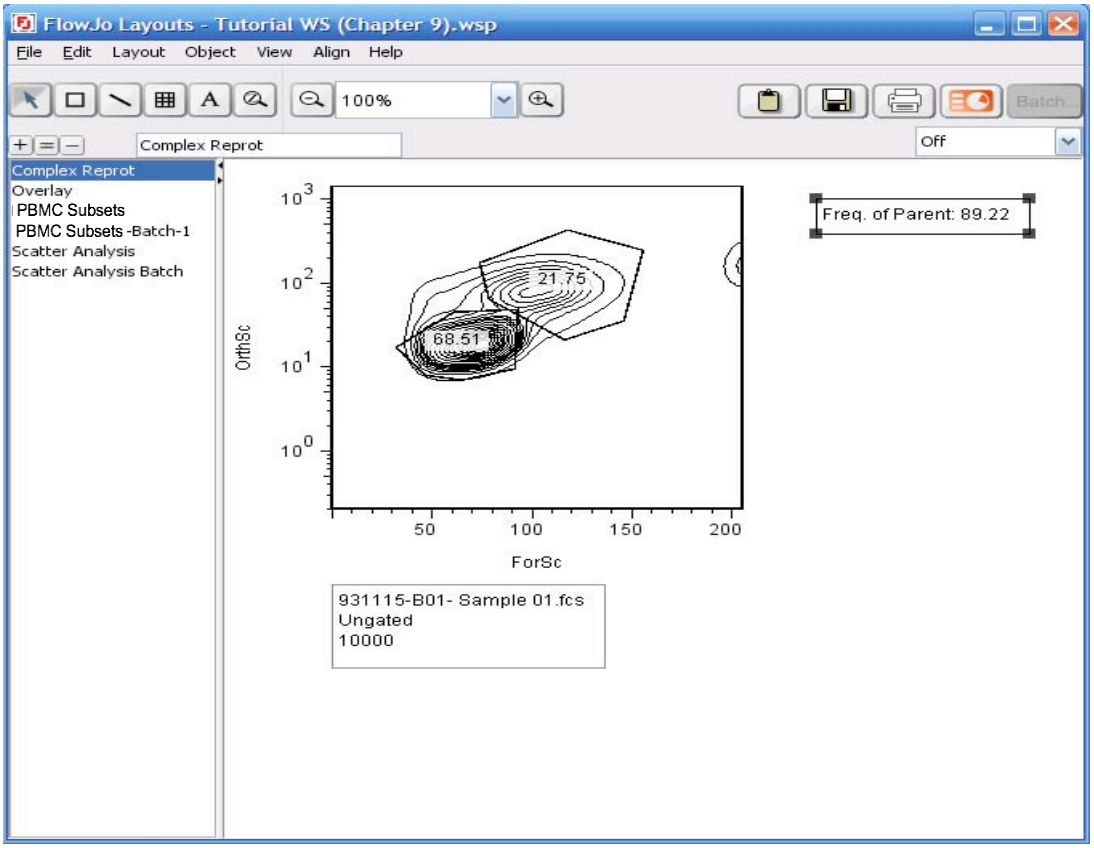


This lesson builds on the Workspace you have finished from Chapter 8; alternatively, you can open the workspace named "Tutorial WS (Chapter 9)". Open the Layout Editor and create a new Layout named "Complex Report." Drag into the Layout View the first ungated sample, **931115-B01...** The Layout View should appear as shown left.

In the ***Workspace***, click on the **Freq. of Parent** statistic under the **CD45+** gate.

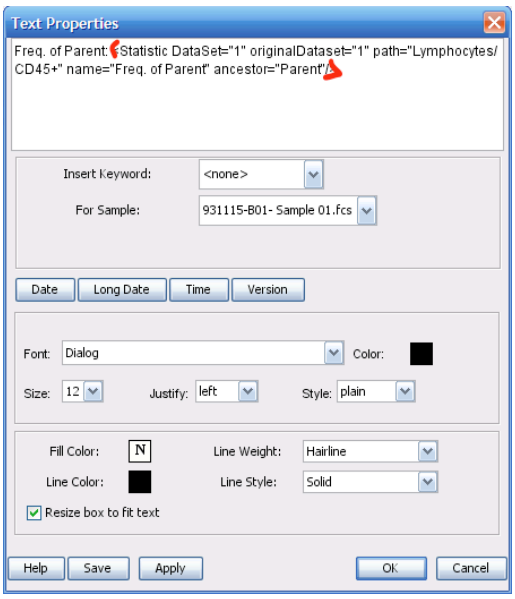
Drag this statistic into the ***Layout Editor***, holding the mouse button down as you move the statistic into the ***Layout Editor***, and drop it (releasing the mouse button) to the right of the graphic. FlowJo creates a text box that shows you the statistic value, as shown in the figure on the next page.

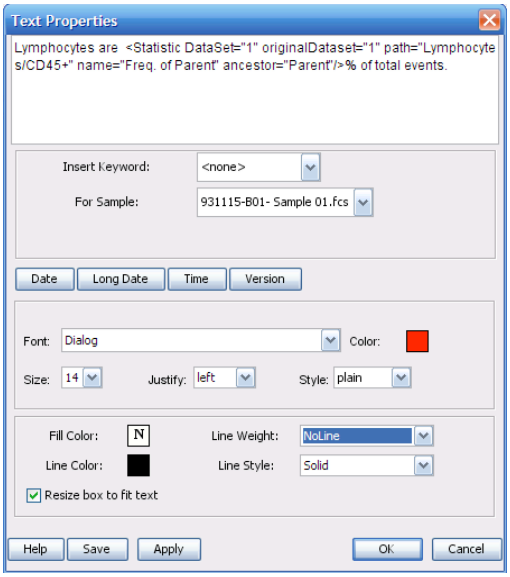




You can add additional annotation to the layout, if you need to. Here we want to add some additional text to this box. To make room, make the text box a little bigger by clicking on the lower right handle, and resizing it to a larger size. Then, double-click on the text box to have FlowJo open the ***Text Properties Window***, as shown below:

Note that the statistic value has been expanded to a bracketed (**<...>**) command. The command within the brackets tells FlowJo exactly how to get the desired statistic (i.e., which subset and which statistic are desired). **DO NOT** edit the text within the brackets, or else FlowJo may not be able to fetch the statistic value you want! However, you are free to edit any of the text outside of the brackets. In this example, select all of the text in front of the first bracket and change it to "Lymphocytes are ", and after the second bracket, add the text "% of the total events". The window should look like shown on the next page:

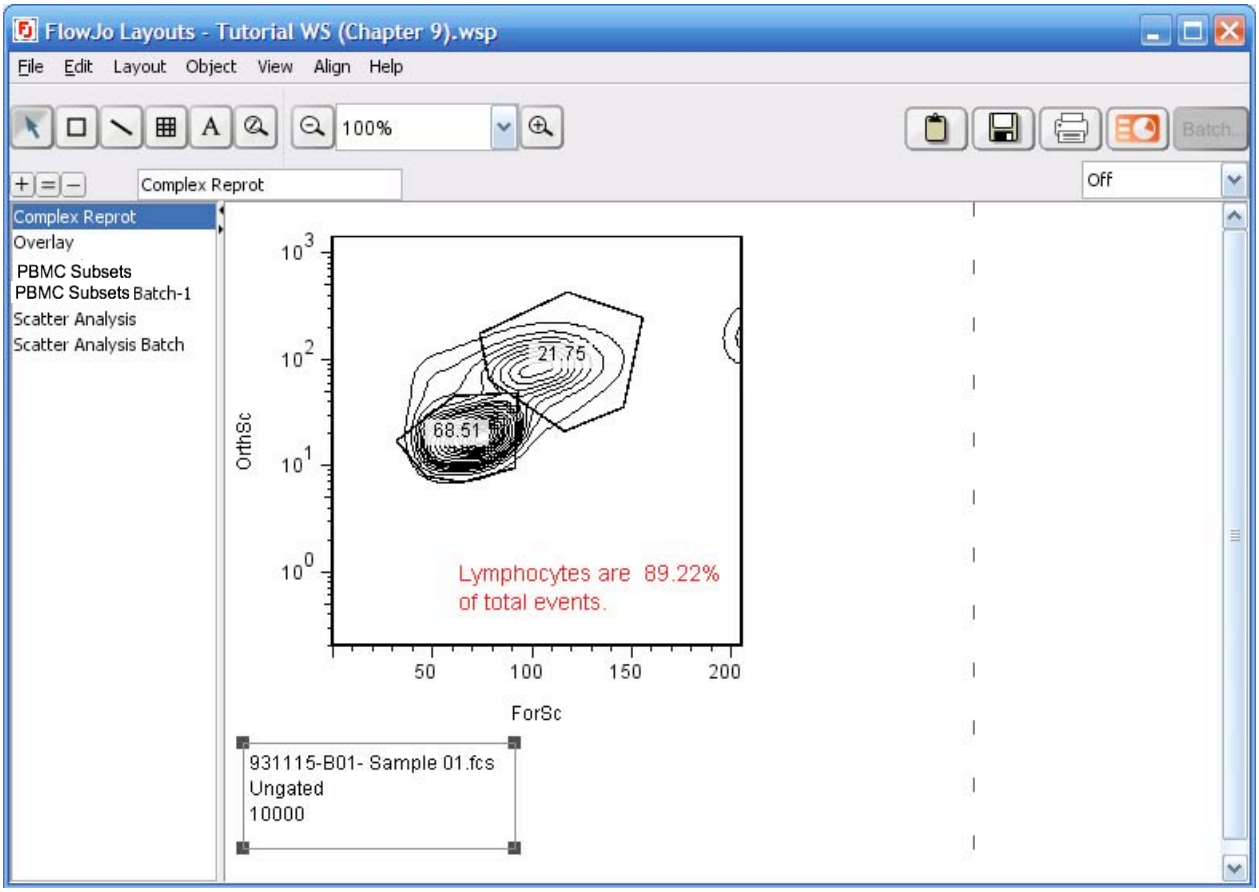




To change the particulars of the text display select a red text color and 14 point font size. You can click on the **OK** button to accept changes and go on.

Note that you can also select keyword values to add to text boxes (the keyword values are data encoded in the text box file itself). You can create additional text boxes with more statistics by dragging them from the workspace. You can also add multiple statistics to one text box: by selecting multiple statistics and dragging them into the Layout View Again, you are free to edit the text outside of the brackets; don't edit text within the brackets.

Now click on the text box, and drag it so it overlays the graphic item in the ***Layout View***. The view should look something like what is shown below.



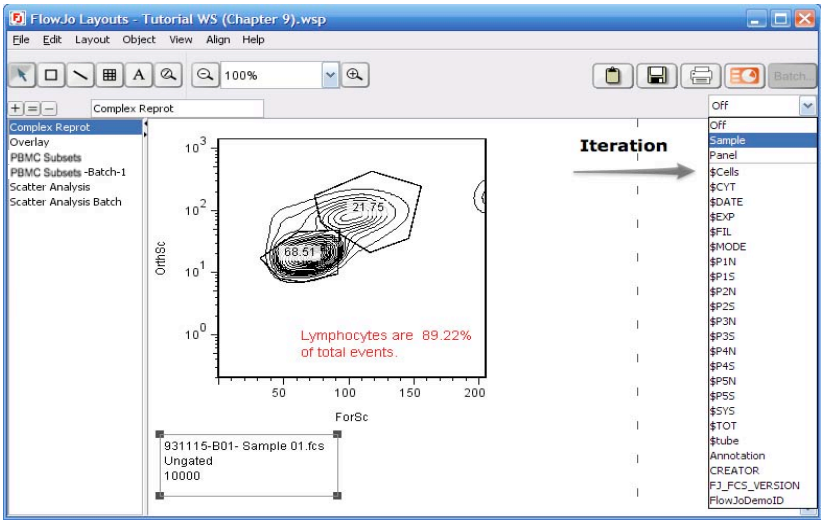
Note that, like the graphs, statistics in the Layout editor are also ***live***. If gates change, causing the number of events to change, then they are automatically updated.

In addition, Statistics respond to the ***Iteration value***; if you select a specific sample to view, then statistics are shown for that sample. (If you select a statistic of a subset that does not exist in the current Iteration sample, then the statistic value is shown as "n/a". Likewise, a graph for such a subset would be shown as a Placeholder).

The final topic in this Chapter deals with Layouts that derive graphs (or statistics) from different samples (tubes). This will only be useful if your data files are properly annotated.

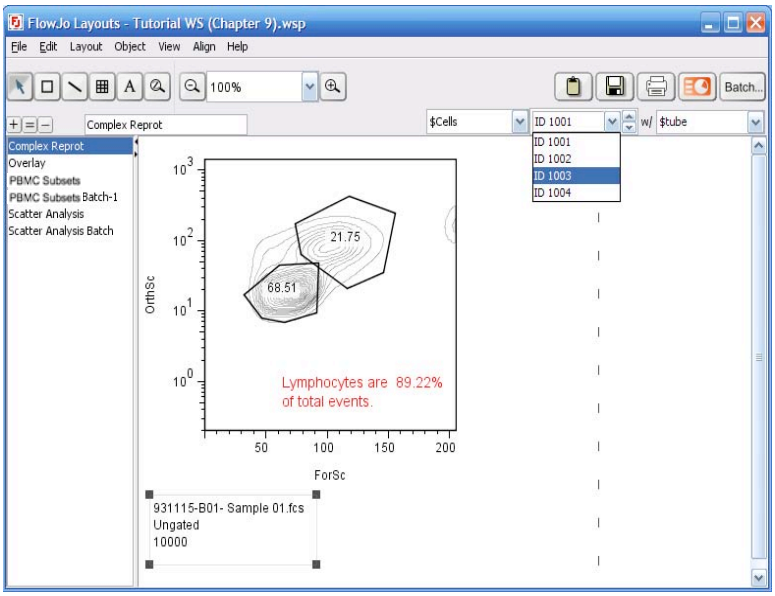
As you learned in Chapter 8, FlowJo forces all graphs to be derived from the same sample during iteration. To create a batch output, FlowJo forces the iteration value to cycle through all possible values (for the current Group). By default, this means one Frame for every sample (tube) in the Group. However, you may want to generate graphic reports wherein each Frame derives graphics from multiple samples. For example, you may want to generate one frame for each patient, and therefore iterate by patient ID. Alternatively, you may want to iterate such that each iteration value corresponds to a different tissue studied from an animal, where you have performed multiple stains on the tissue samples. In these cases, you would like to Iterate not over samples (or tubes), but to iterate over Patients or Tissues.

If your FCS data has keywords that contain such information, then FlowJo gives you this ability. If you don't know how to add this information to your data, ask your local Flow Cytometer operator or Instrument Sales Representative. For the demonstration in this tutorial, the data supplied has a Keyword Field, $Cells, which has a unique value for each different Patient sample. In the demonstration data, there were four patient samples (**ID0001 to ID0004**) that were stained with four different combinations of antibodies. The Experiment 2 Folder has Patient ID0005 through ID0018 which you may wish to analyze later as a larger data set.



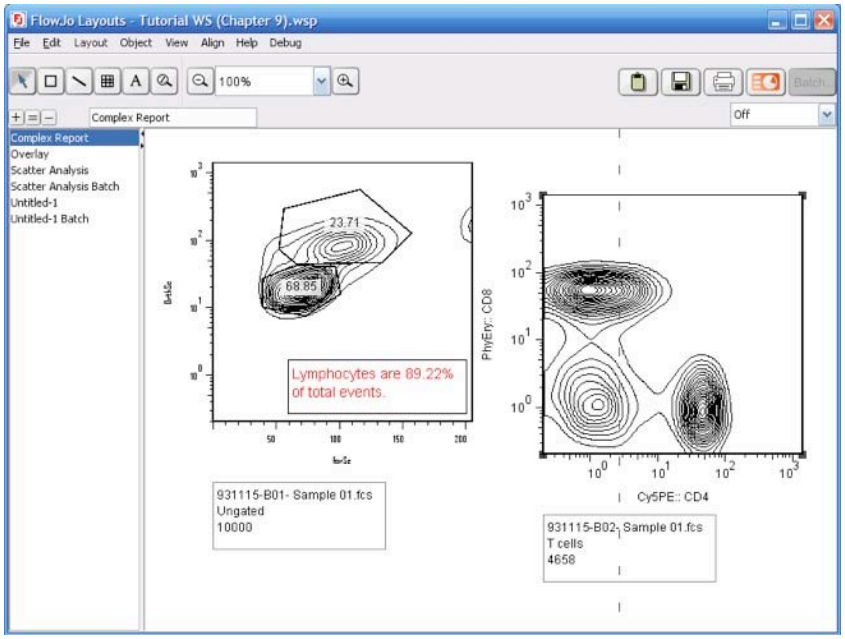
We will now tell FlowJo that it should iterate over patient samples. FlowJo has a menu where you can specify which FCS keyword should be used as the controller for Iteration. Select **$Cells**, which, for this dataset, contains the unique patient ID.

After you choose the iteration parameter, FlowJo will search through every sample in the current Group, and build a list of unique values of the Keyword **$Cells** found in the current group. The current workspace has four unique values of $Cells which are shown by clicking on the iteration menu (see image to the right).



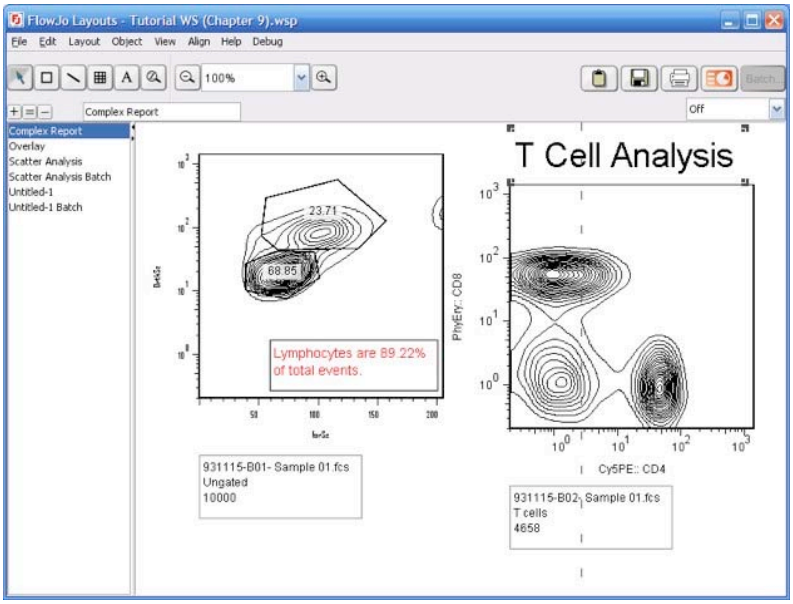
It is further possible to distinguish between samples matching in all of three criteria by determining a discriminator keyword in the iteration options. In our example this would be the **$tube** keyword which is different for each staining used (PBMC, T-cells, CD4 cells, and CD8 cells), thus allowing us to unambiguously identify a sample by the **$Cells** and **$tube** keywords. Select $tube as the discriminator as shown above. Now you can select the different patients and have the graphs and statistics update in the layout view.

Set the ***iteration value*** back to **Off**. This specifies that FlowJo will only show you the graphs and statistics from the originally dragged subset.



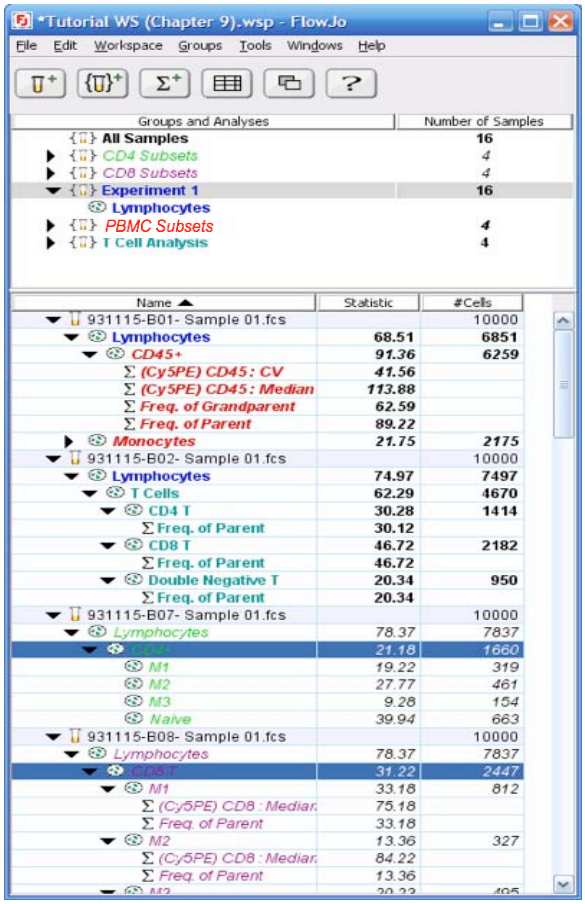
The graphs and statistics shown in the Layout View were derived from the first antibody combination (CD14/CD16/CD45). Now we will drag in a graph from a different staining combination. Select the **T cells** subset from the sample **931115-B02...** and drag it into the Layout Editor next to the first graph. Your **layout** should appear as depicted to the left.

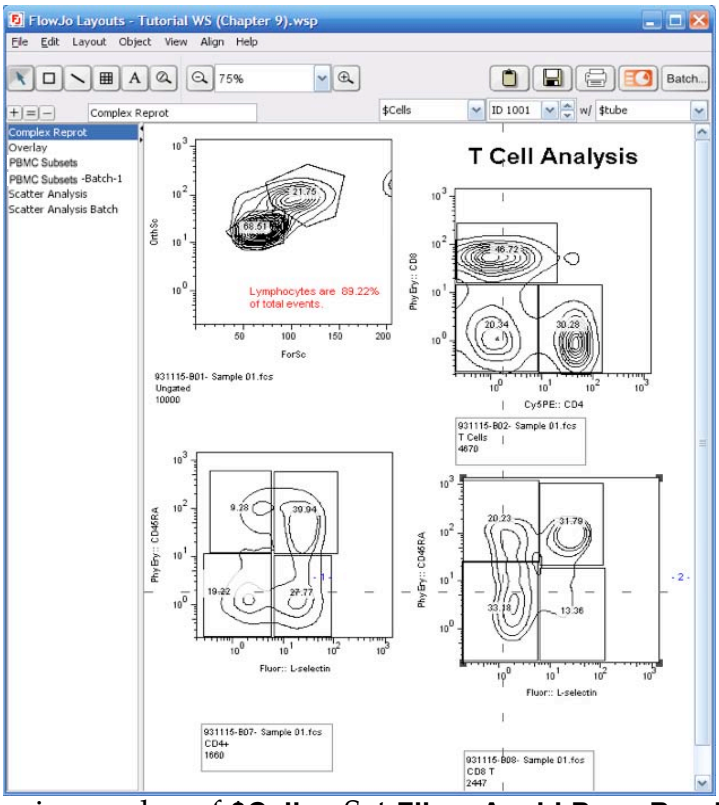
Add a text box item above the second graph, typing in “T Cell Analysis”. Select the text and setting the font size to 36 points. Set line color to none and click OK. The Layout View will look something like the next image:



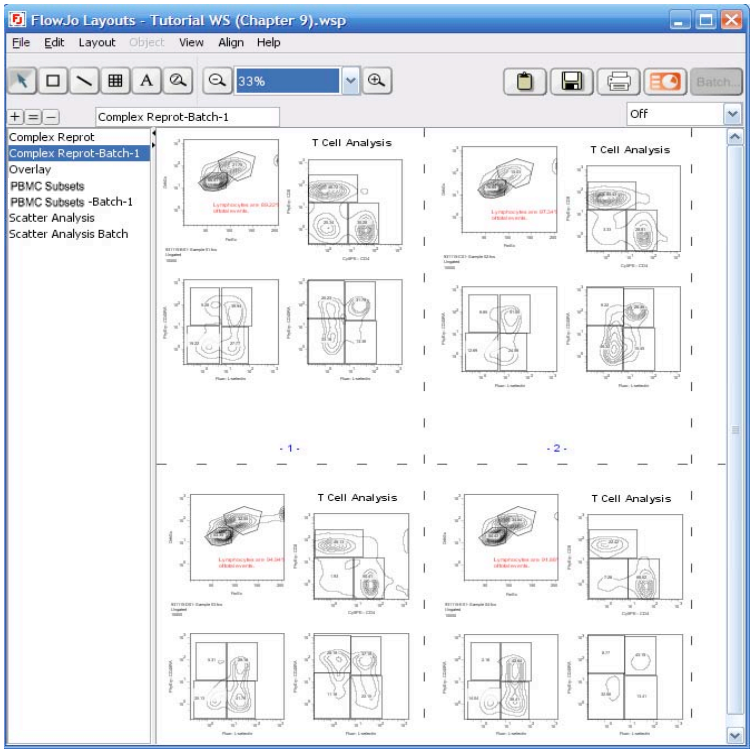
Make the ***Layout Editor*** Window larger (by dragging the lower right hand corner to resize the window), in order to make room for more graphs in the Layout View.
Back in the ***Workspace*** Window, click on the “CD4 T” subset and ***control***-click the “CD8 T” subset under the next two samples (as shown to the right).

Click on one of these and drag (both will be dragged) into the Layout View, just below the first graphic. Now two more graphs appear. Adjust them as shown next.





Select the annotation text boxes under the three newest graphs, and press the ***delete*** key to remove the annotations. Move the graphs a little closer together Now you have created a graphical report that draws graphs from different tubes of the same patient. Now set up the iteration by selecting $Cell as the iterator and $tube as the discriminator as shown to the left. Change the patient ID to "ID 1004". All four graphs and the statistic change, and now are drawn from the fourth patient sample in the workspace. You can select any patient sample to view the graphs. To view a complete report, just click on the ***batch generation button***. Set the geometry to 2 columns and generate a new Layout. FlowJo now iterates (only four times!), and generates four ***tiles***: one for each unique value of **$Cells.** Set **File > Avoid Page Breaks**. You should see a window such as the one below.



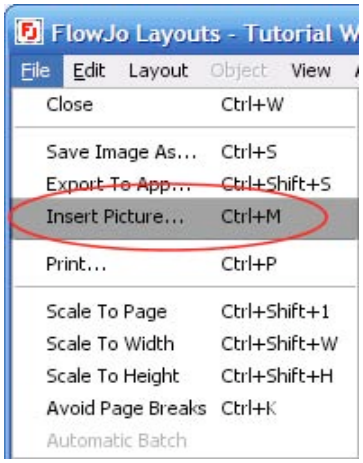
You may wish to experiment some more by dragging in the folder for **Experiment 2** on to the current workspace, and regenerate these layouts. They will be considerably more complex, as you will have 18 patient samples; however, you will quickly appreciate how little additional work is involved in your future analyses!

In the next Chapter, you will learn some additional Layout Editor techniques, including creating Grids and placing background graphics underneath the reports.

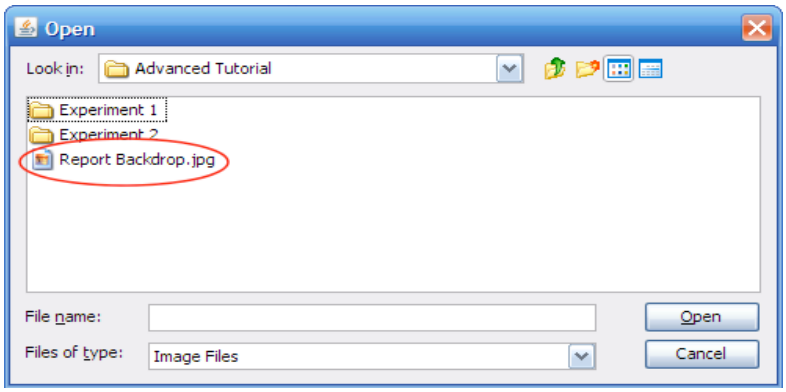
Lesson 10: Creating Finished Reports

In this chapter, you will learn how to use some of the advanced features of FlowJo's Layout Editor to create reports. You will learn how to create live text objects that contain sample-specific information and statistics, how to put in a backdrop containing (for example) logos or specialized forms, and how to manipulate Layout Grids – specialized tabular elements that can contain text, tables, graphs, or any other items.

This lesson builds on the Workspace you have finished from Chapter 9; alternatively, you can open the workspace named **Tutorial WS (Chapter 10).** Open the ***Layout Editor,*** and create a new Layout named **Final Report**. Select **66%** in the scaling popup menu (at the top of the window), chose View menu from the top menu bar and select "Show Page Breaks" so that you can see the outlines of the page breaks.

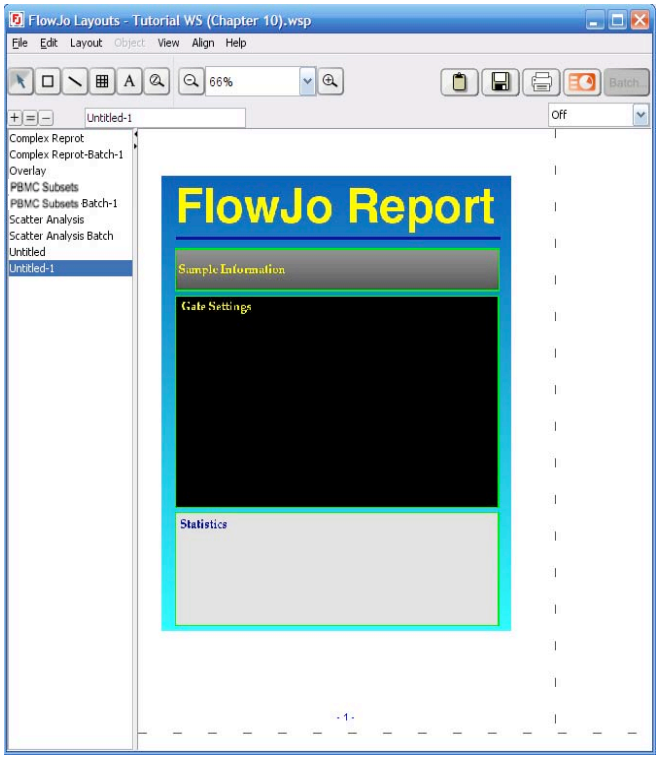


First, add a backdrop. From the **File Menu**, select **Insert Picture...** Navigate to the Advanced Tutorial Folder>PC Workspaces select "Image Files" from the popup menu, and open the file "Report Backdrop.jpg".



FlowJo adds the image to the layout. This will be the template upon which you will add other elements (like statistics and graphics) in order to create the finished report. The layout window should appear as shown on the next page.

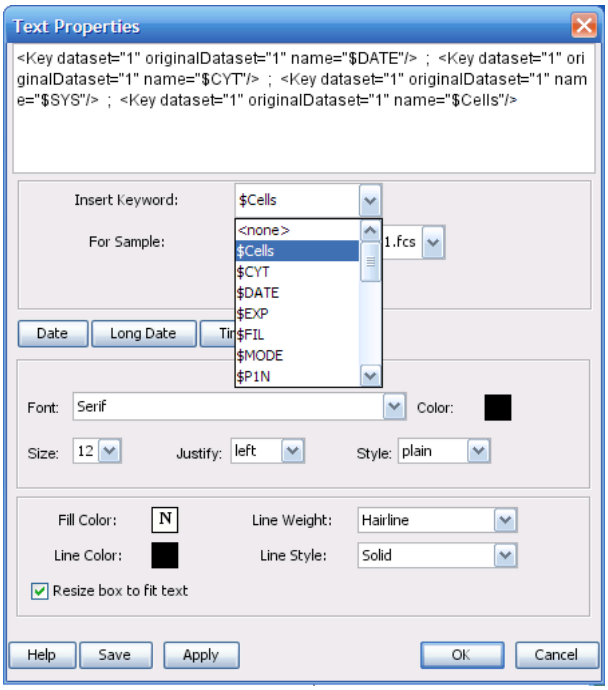
For your own reports, you can generate whatever design you wish – including specific areas for text, graphics, signatures, etc. Simply design the form in any graphics program and save it and open it as you did this one.



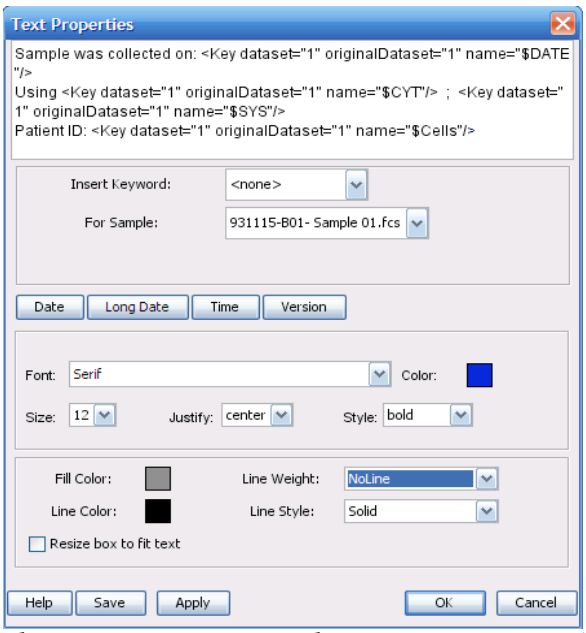
In this report, you will include three separate elements. In the top panel you will create a text box containing information regarding the sample. In the second panel, you will put graphs that show the gating scheme used; finally, the bottom panel will contain statistics and graphs.

Because this layout will draw on information from multiple tubes for the same individual, you will need to set the Iteration Options to **$Cells** for the iterator and **$tubes** for the discriminator. (If you don't remember how, see the second half of Chapter 9).

Begin the first panel by clicking on the text box tool (the **A** button at the top of the window), then click-and-drag a rectangular area that will fit to the right of the **Sample Information** text and within the gray area. FlowJo brings up the **Text Properties** window. Here you will select several keywords that contain sample-specific information for entering into the text box. To insert a keyword value, simply select it from the popup menu (as shown). Begin by selecting the keywords **$DATE** (date of sample collection), **$CYT** (cytometer used for collection), **$SYS** (the system on which data was acquired), and **$Cells** (the sample identification). In your own datasets, you may have other keywords with important information that you can add.



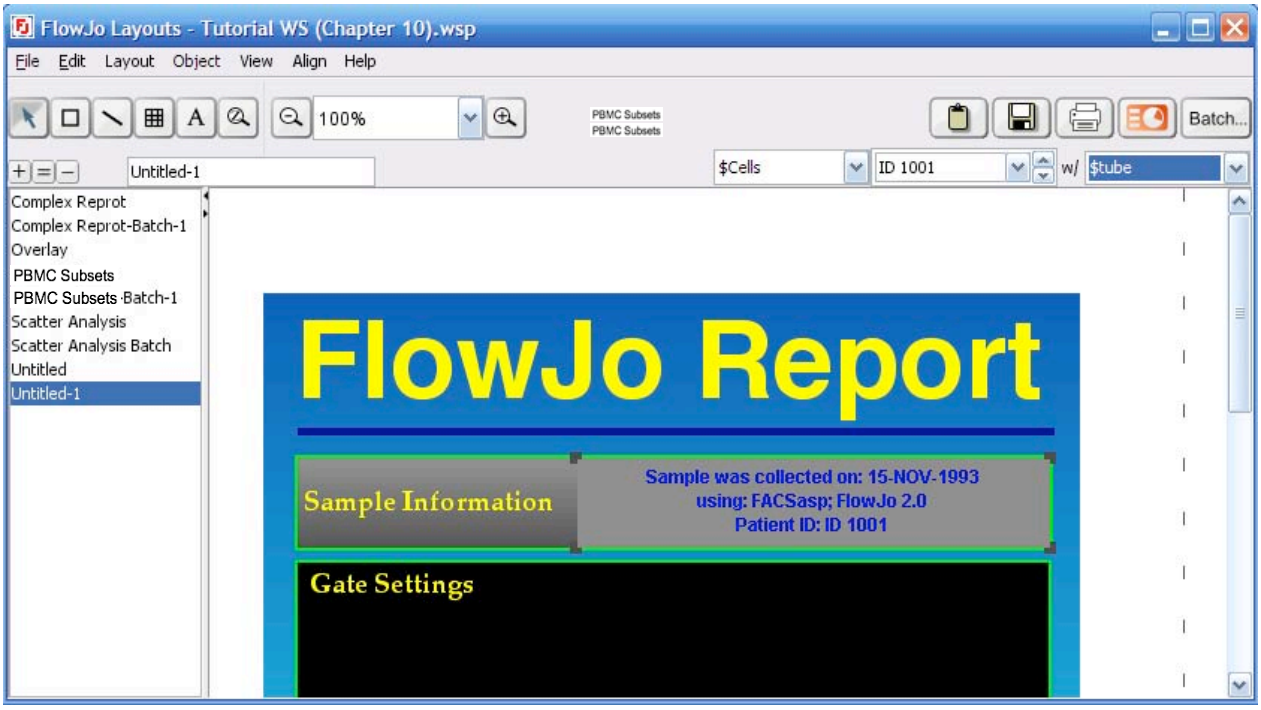
Each keyword command is bracketed <…> Do not edit any text within these brackets. However, you can add text between sets of brackets. As shown in the next image, you can type in explanatory text (and hit return to create line breaks) to format the text box. Set the Fill Color (background color) to gray, the text color (called simply "Color") to blue, and the Style to bold and Justify to centered.



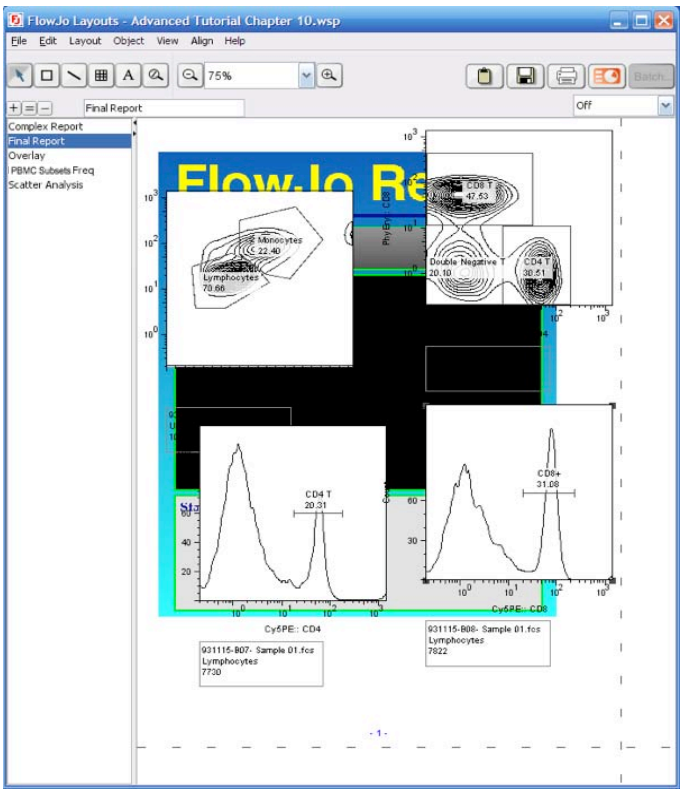
Note that the Fill Color applies to the line drawn around the text box, should you choose Line Weight**: No Line**. When you are finished, click on **OK**.

In the ***Layout Editor***, magnify the view to 100%. The ***Layout Editor*** should look something like that below. If you wished to have a particular keyword value in a different font or color, you would have to make a separate text box for it and format it accordingly.

The next job is to add plots into the middle panel graphics that show how each antibody staining panel was gated. This is similar to some of the layouts you created in previous chapters. First, drag the parent sample (931115-B01-Sample01.fcs) from the Workspace into the ***Layout Editor***, dropping it over the Gate Settings panel. You will note that you cannot see the text, because the graphic text is black. We will change this momentarily.

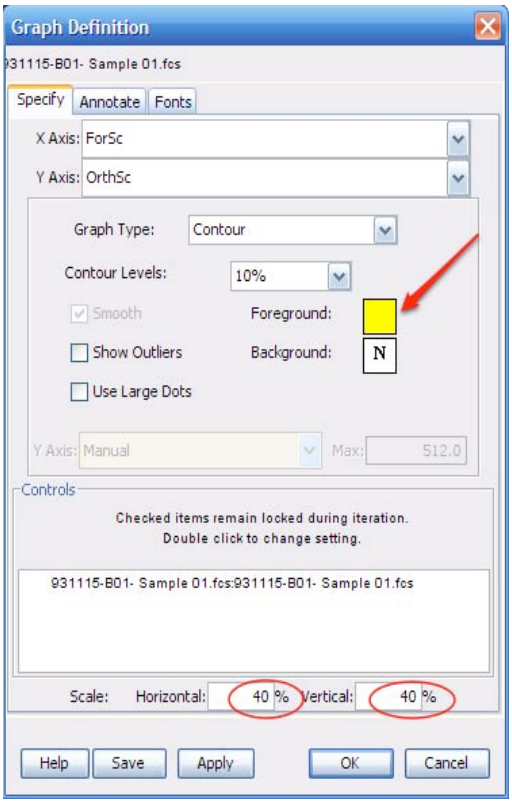


However, first drag in three other graphs into the ***Layout Editor*** as shown on the next page; select the **T Cells** graph from the second sample, and the two **Lymphocytes** gates from the first sample within the **CD4** **&** **CD8** groups in the Workspace. Drag them all onto the ***Layout Editor*** one by one. Your view will probably look something as shown on the next page.



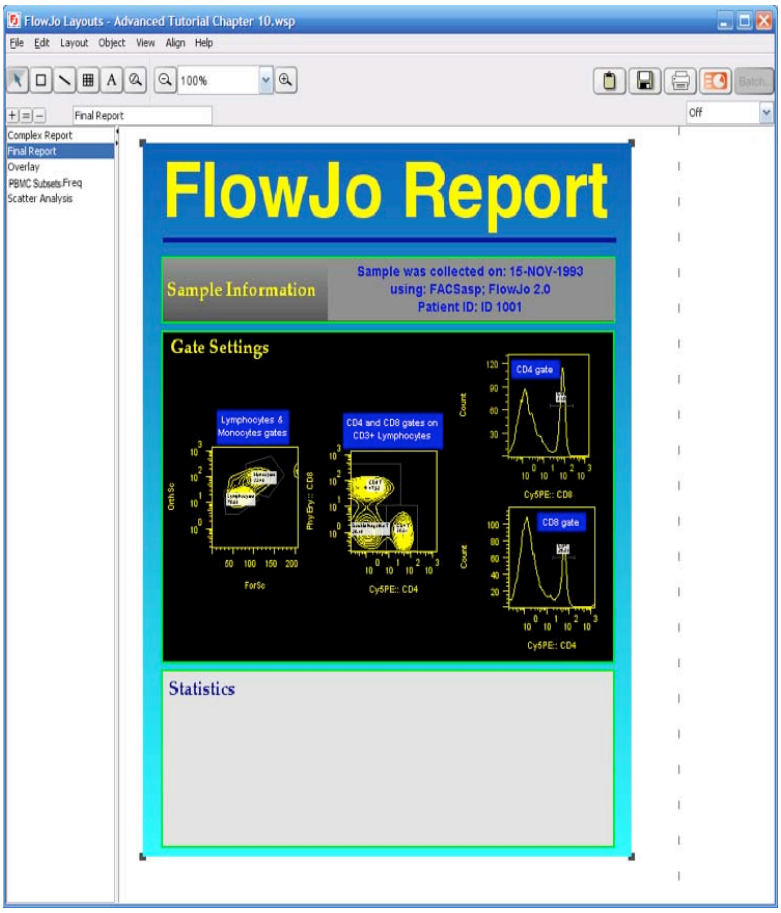
You will now change the attributes on these four graphs. Double click each graph in turn.

As shown in the window below, change several attributes. First, change the foreground to yellow, the background to none and type in values of **40** in the two scaling boxes. Next, click on the annotate tab to uncheck Show Annotation (a check mark means that the attribute is On; a blank box means the attribute is Off). In the Fonts tab change the Foreground font to yellow.



When you are done, click on the OK button. All four items are changed as you requested.

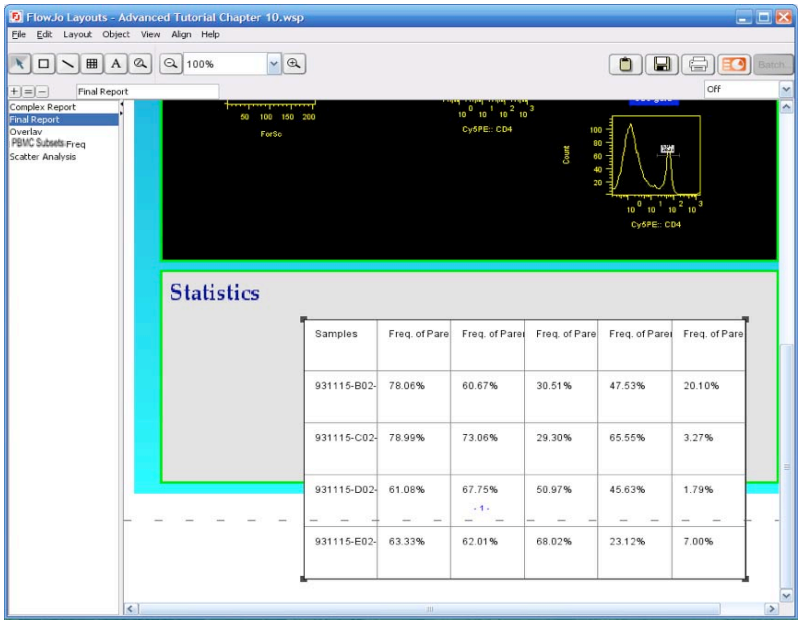
Now move the graphs into different locations, perhaps as shown in the example on the next page. (the lymphocyte and monocyte gates are shown first, then the CD4 and CD8 gates on the CD3 subset, and on the right, the CD4 and CD8 gates). Note that each graph is derived from a different tube. The graphs may appear very dim at low magnifications; however, they will print just fine (and, if you scale up to 200% or greater, you can see that they are drawn fine at high resolution).



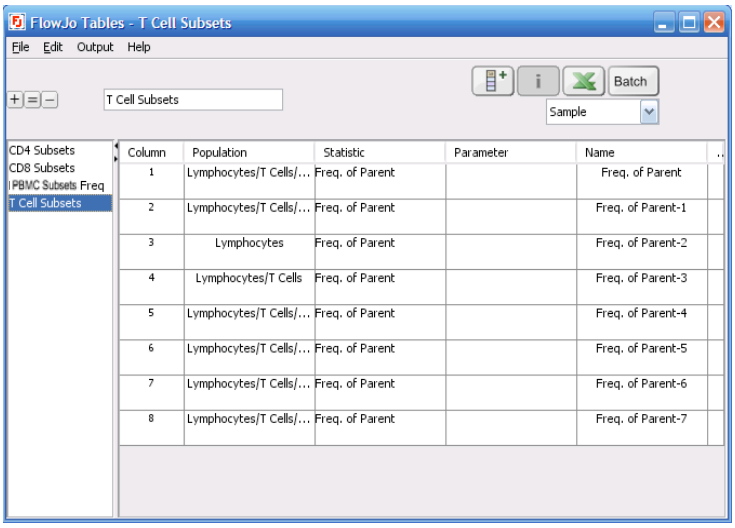
Finally, you can create some text boxes to annotate the graphs. In this example, the new text boxes were created with a white text overlaying a dark-blue background. Tip: create one text box; format it exactly how you want. You can then duplicate it (using copy-paste, or right-click **Duplicate**). Double-click on the text box to change its contents.

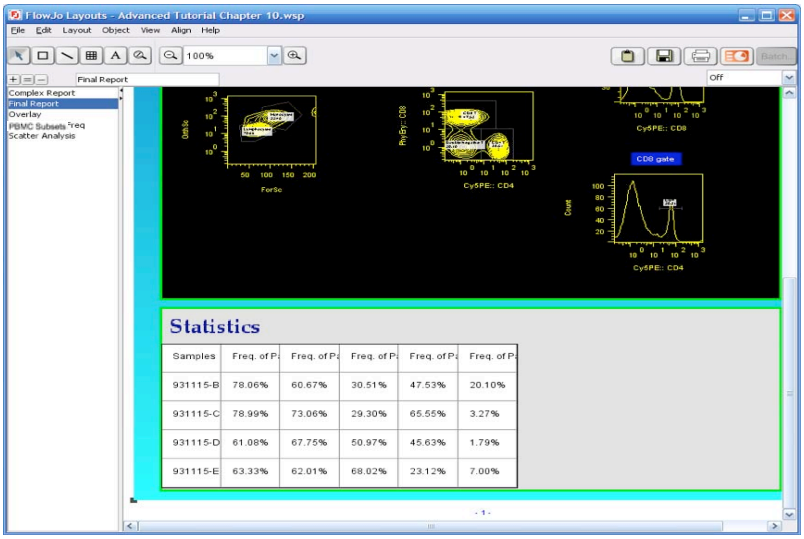
For the final panel, we will create two different tables. The first table will have just statistics; the second table will mix statistics and graphs.

In the table below, you see in the "Final Layout" was generated by adding a FlowJo table. Open the Table Editor and select your previously created, "T Cell Subsets" table. Batch to create the table and add to Layout as you did in Lesson 6. You should see the table in the Layout Editor.



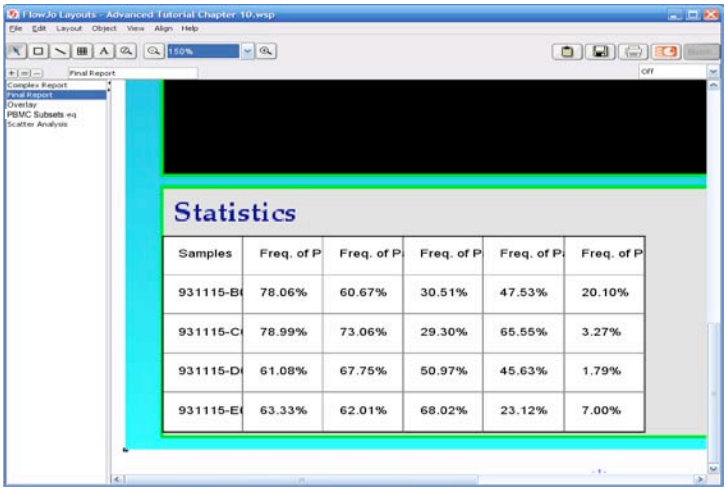
Next, resize the table to make it smaller. Click on the lower right hand selection handle and drag it to make the table fit in the area of the ***Layout Editor.*** You can not resize individual rows and columns by clicking on any dividing line and dragging.


FlowJo Tables - T Cell Subsets
File Edit Output Help
T Cell Subsets
Batch
Sample
CD4 Subsets
CD8 Subsets
PBMC Subsets Freq
T Cell Subsets
Column Population Statistic Parameter Name
1 Lymphocytes/T Cells/... Freq. of Parent Freq. of Parent
2 Lymphocytes/T Cells/... Freq. of Parent Freq. of Parent-1
3 Lymphocytes Freq. of Parent Freq. of Parent-2
4 Lymphocytes/T Cells Freq. of Parent Freq. of Parent-3
5 Lymphocytes/T Cells/... Freq. of Parent Freq. of Parent-4
6 Lymphocytes/T Cells/... Freq. of Parent Freq. of Parent-5
7 Lymphocytes/T Cells/... Freq. of Parent Freq. of Parent-6
8 Lymphocytes/T Cells/... Freq. of Parent Freq. of Parent-7

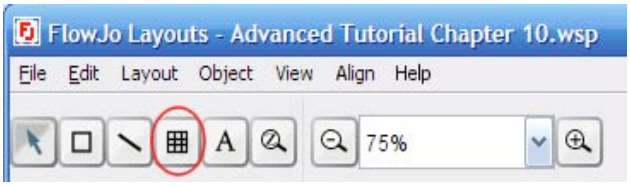


Resize the table so that most of the text is shown in each cell (left).

If you increase the magnification on the ***Layout Editor,*** you should see your table similar to the one show here.

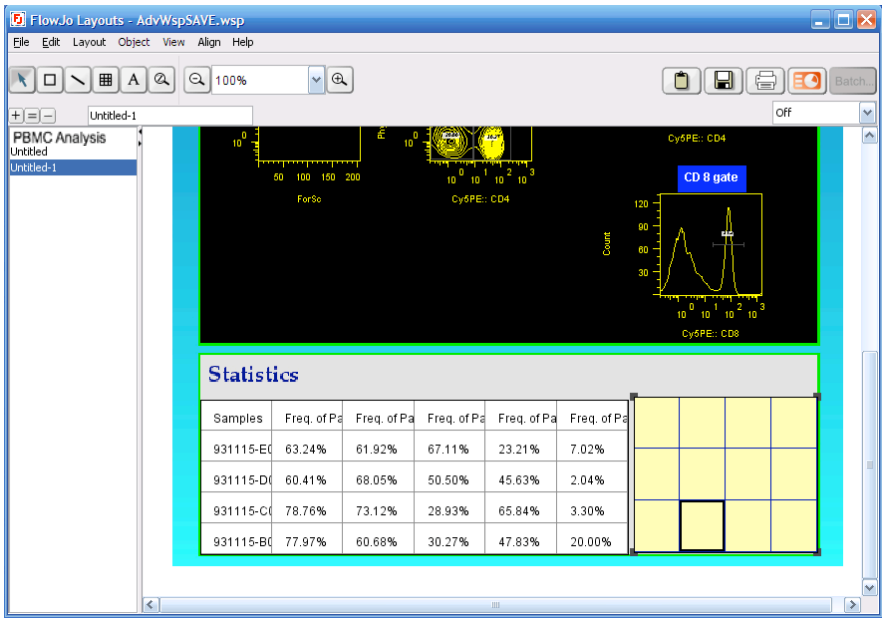


Now it's time to create a Grid. In this Grid, you will have three rows: one title row, one for CD4 T cells, and one for CD8 T cells. As well, you will have 4 columns: the first is a title, the second will have the Median CD3 fluorescence on the specified subset, the third will have the percent of CD3 T cells that are CD4 (or CD8), and the fourth will have a histogram of the CD3 intensity for the gated subset.

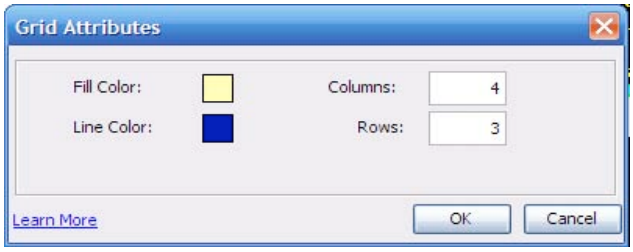


To create a blank Grid, click on the Grid tool icon (it is the fourth from left at the top left edge of the layout window). Click and drag a rectangle that occupies the right portion of the Gray Statistics panel of your layout view. (You may have to adjust the magnification in order to see the full area of interest).

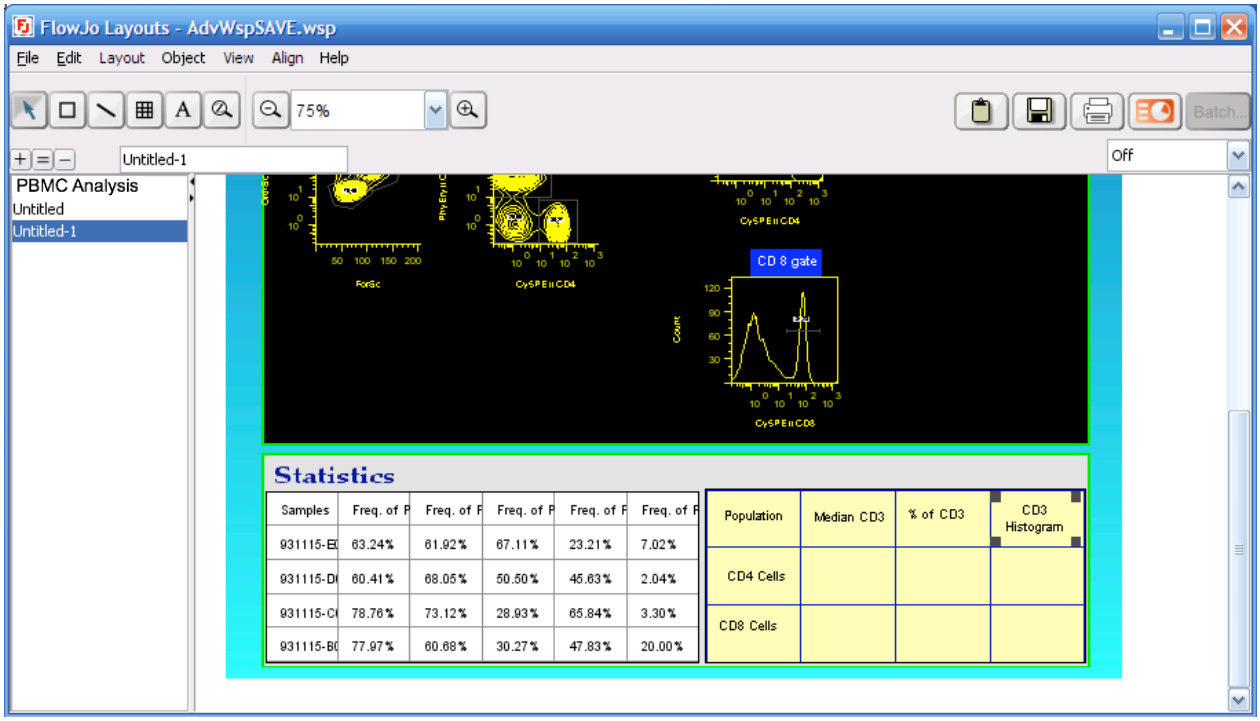
FlowJo now shows the empty Grid placed in the ***Layout Editor***.



Double clicking on the grid will bring up the Grid Attributes window. Select a light yellow Fill Color and a dark blue Pen Color and enter the dimensions of 3 rows and 4 columns.

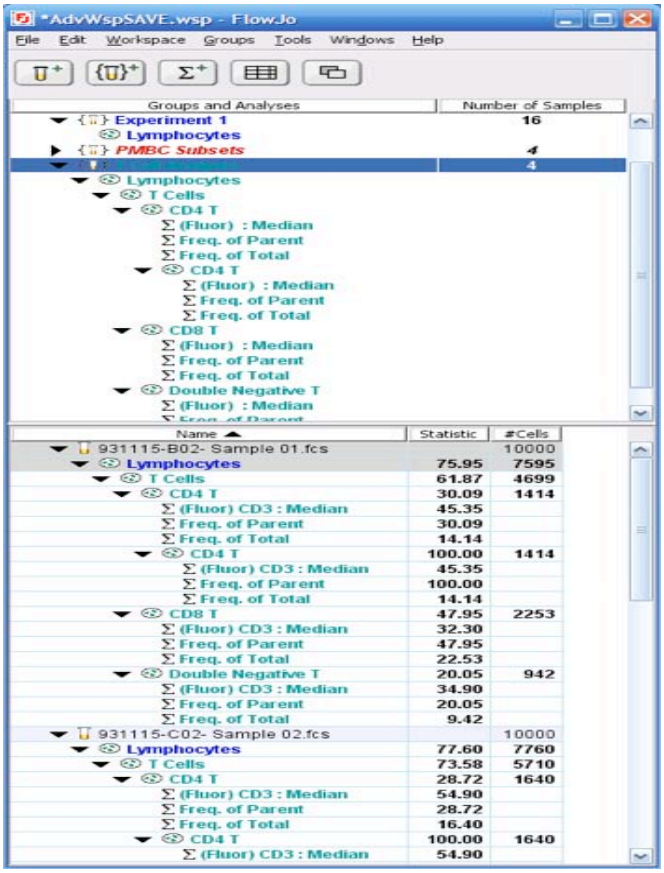


Finally, it is time to start annotating the Grid contents. Use the Text tool to add the titles Population, Median CD3, % of CD3, and CD3 Histogram to the first four cells in the top row. Add the titles CD4 cells and CD8 cells to the second and third cells of first column. Set the text attributes to bold text. Your ***Layout Editor*** may appear as shown below.



Note that any item in a Grid can be moved out of the Grid or to another position: just right click on the item (so the cell is selected), and copy/paste it to the new location! Likewise, you can take any item from that Layout View, and drop it onto any empty cell in the Grid to make it part of the Grid. To add graphs and statistics to the Grid, you will select them from the Workspace, and drop them into the appropriate positions in the Grid.

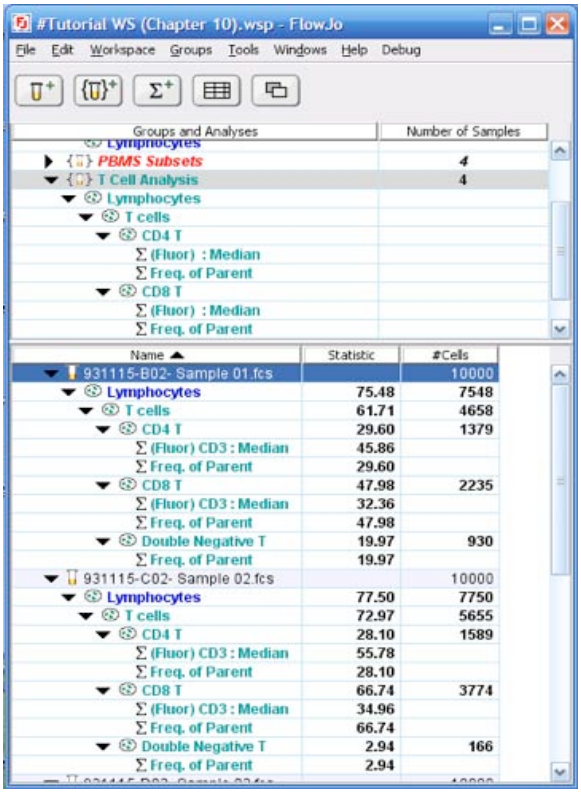
First create the appropriate statistic. In the Workspace, select the "T Cell Analysis" Group. To a CD4 gate within the first sample, add the statistic "Median Fluor:CD3". Drag this statistic to the Group's CD4 and CD8 gates so that it is applied to all samples in the workspace. Your Workspace window should appear as shown in the next page:

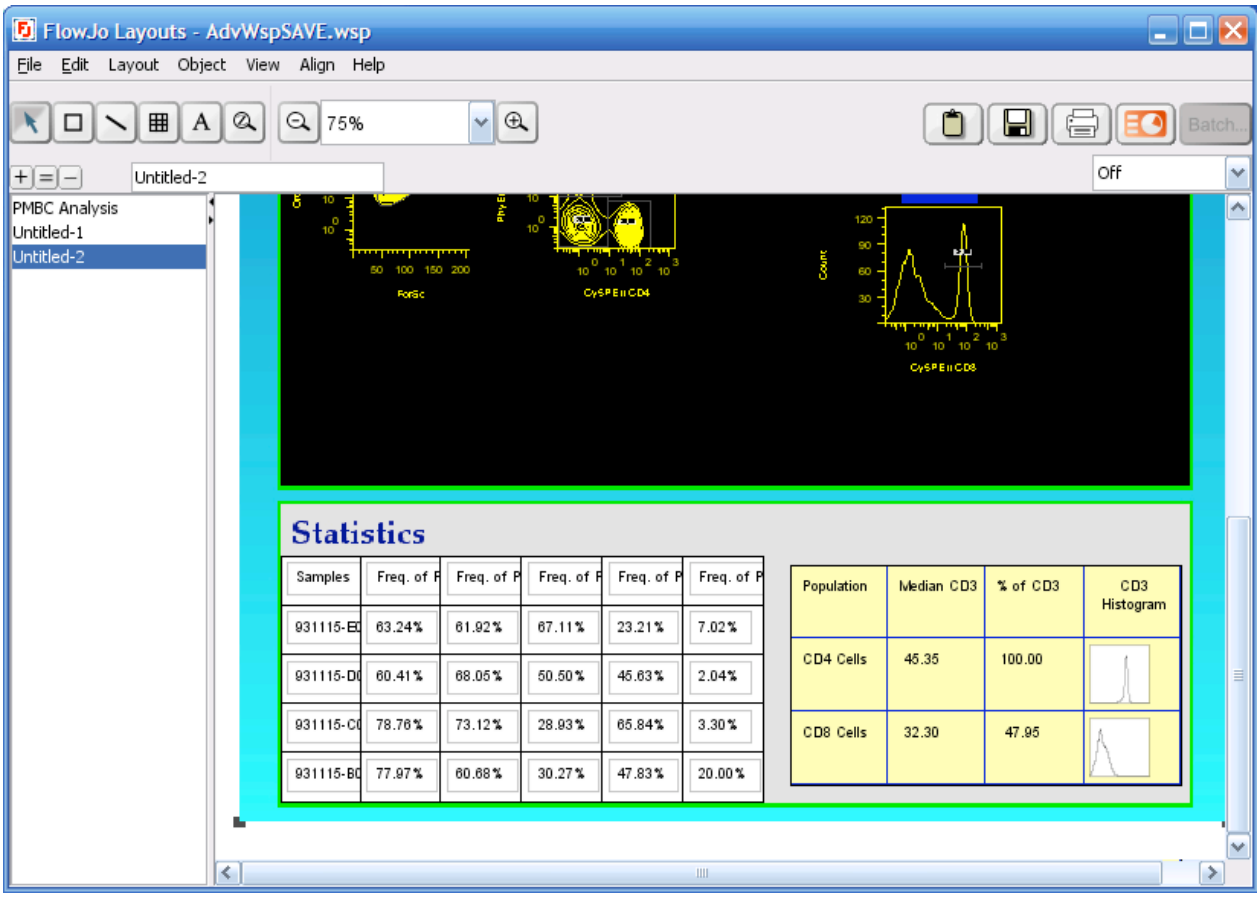


Now click on the Median CD3 statistic under the CD4 gate, and drag it to the second column of the second row of your new Grid (drop it when that cell is highlighted). FlowJo creates a new text box with the statistic and adds it to the Grid. Add the **Freq. of Parent** statistic to the next column. Repeat the two drags for the statistics bound to the CD8 subset into the third row of the Grid.

If you wish, you can double click on each item individually (make sure that only one Grid cell is selected) in order to edit the text: remove the annotations from the Text to leave only the statistic for each cell.

To add graphs to the Grid, click on the population of interest (from the Workspace) and drag it into the correct cell of the Grid (Or, if you already have the graph in the Layout Editor, you can drag it and drop it into the Grid). Select the CD4 subset and drop it into the last column; likewise for the CD8 subset. To change the graphs to histograms of CD3, select the two cells with graphs, and double-click. Change the X-Axis to "Fluor CD3" and the graph type to Histogram. At this point, your Layout Editor should look something like that shown in the next page.

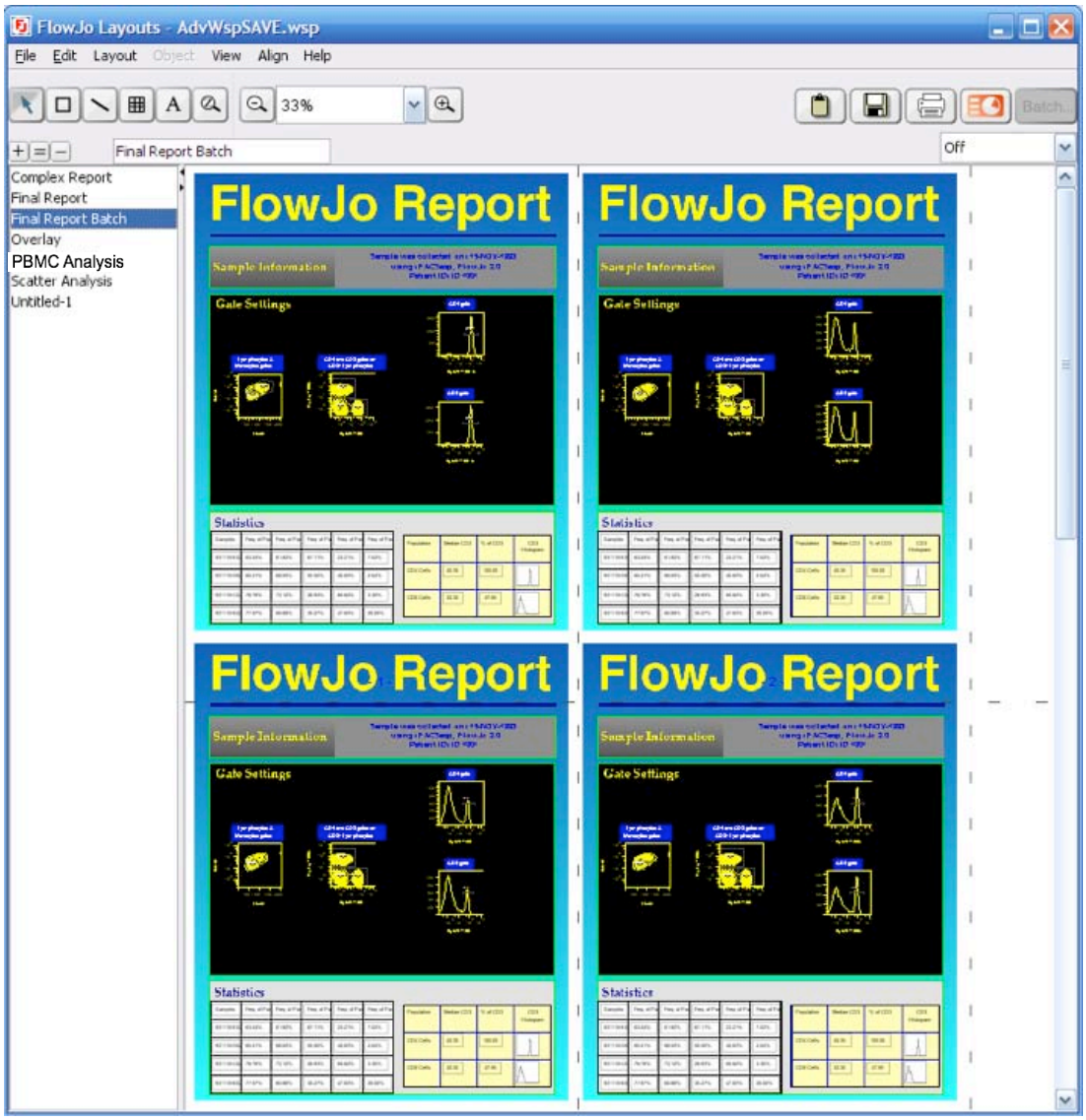

#Tutorial WS (Chapter 10).wsp - FlowJo
File Edit Workspace Groups Tools Windows Help Debug
Groups and Analyses
Number of Samples
PBMS Subsets 4
T Cell Analysis 4
Lymphocytes
T cells
CD4 T
Σ (Fluor) : Median
Σ Freq. of Parent
CD8 T
Σ (Fluor) : Median
Σ Freq. of Parent
Name
Statistic
#Cells
931115-B02- Sample 01.fcs 10000
Lymphocytes 75.48 7548
T cells 61.71 4658
CD4 T 29.60 1379
Σ (Fluor) CD3 : Median 45.86
Σ Freq. of Parent 29.60
CD8 T 47.98 2235
Σ (Fluor) CD3 : Median 32.36
Σ Freq. of Parent 47.98
Double Negative T 19.97 930
Σ Freq. of Parent 19.97
931115-C02- Sample 02.fcs 10000
Lymphocytes 77.50 7750
T cells 72.97 5655
CD4 T 28.10 1589
Σ (Fluor) CD3 : Median 55.78
Σ Freq. of Parent 28.10
CD8 T 66.74 3774
Σ (Fluor) CD3 : Median 34.96
Σ Freq. of Parent 66.74
Double Negative T 2.94 166
Σ Freq. of Parent 2.94



Now you are ready to generate the full report for all samples. Make sure to set the Iteration Options to $Cells for the iterator and $tubes for the discriminator and remember to select the All Samples group first (any batch layout operation is only performed on the current group). Click on the ***Batch*** button.

FlowJo compiles all of the graphs, statistics, text, grids, and backgrounds and puts them into a single window. From here you can print a report, publish to the web, or generate a slide presentation.

Most importantly, your layout is now ready for the next experiment: all you have to do is add the samples to the ***Workspace*** and run the Batch processor: the report will be generated without having to re-generate the layout template! And you can have as many of these templates as you wish for any ***Workspace***.



This ends the tutorial. There is more documentation available in the reference web pages, which you'll reach from any of the ***Help*** buttons within the program, by typing F1 anywhere in FlowJo, or by looking at:

http://www.flowjo.com/v76/en/windowstoc.html

If you have any questions, or ideas for improvements, please contact us at:
flowjo@treestar.com

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Revision Date: February 19, 2010
Version 7.6